THE UNIVERSITY OF ALBERTA

GROWTH OF CANDIDA UTILIS
ON RAPESEED OIL MEAL HYDROLYZATES

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

FALL 1972

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled GROWTH OF *CANDIDA UTILIS* ON RAPESEED OIL MEAL HYDROLYZATES submitted by G. EDWARD PHILLIPCHUK in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

At present, rapeseed is grown primarily for the production of edible oil; rapeseed oil meal (RSOM) constituting a waste of increasing magnitude. The meal, which is the residue remaining after oil extraction, consists mainly of protein and carbohydrate; however, potential animal feed utilization has been hindered by problems associated with palatability, digestibility, and toxicity of the meal.

The study involved degradation of rapeseed oil meal by acid hydrolysis (6N HCl) and enzymic digestion (pepsin, trypsin) into a form capable of utilization by *Candida utilis* 9950. Acid hydrolysis and enzymic digestion of rapeseed oil meal resulted in RSOM-preparations varying in chemical composition and suitability to support microbial growth. Nutritional studies using *Candida utilis* 9950 showed that substitution of conventional nitrogen sources in commercial media formulations by RSOM-preparations at equivalent nitrogen concentrations ($\geq$ 1000 mg nitrogen/l) was impractical due to problems associated with precipitation of medium components during sterilization; however, lower nitrogen concentrations (110 mg nitrogen/l) and equivalent carbohydrate supplementation yielded growth response comparable to Sabouraud Dextrose Broth, Tryptic Soy Broth and Czapek-Dox Broth but inferior to Mycological Broth. Pepsin hydrolyzates of RSOM with added carbohydrate supported poor growth of *Candida utilis* at 100 mg nitrogen/l but comparable cell yields were attained when nitrogen concentration of the medium was

increased to 500 mg nitrogen/l. Hydrolyzates prepared by 6N HCl "reflux" method and 6N HCl "autoclave" method demonstrated similar chemical composition and growth response. In addition, a fifty-fold reduction in hydrolysis time was achieved. The study demonstrated the suitability of RSOM-preparations as a microbial medium constituent independent of undesirable components.

ACKNOWLEDGEMENTS

I wish to thank Dr. H. Jackson for his excellent guidance and encouragement without whose help this manuscript could not have been completed. Special thanks must go to Dr. J. Sumner for his supervision and interest during Dr. Jackson's sabbatical leave.

Sincere appreciation is expressed to all academic and non-academic staff of the Department of Food Science for their assistance throughout the study. In particular, I wish to thank Mr. S. Ujvarosy and Mr. D. Jericevic for their technical assistance.

I am also grateful to Dr. L.B. Smillie, Department of Biochemistry, for making the Beckman amino acid analyzer available and Mr. J.M. Nattriss for his care of the amino acid analyses.

Financial support from the University of Alberta in the form of Graduate Teaching Assistantships and the Alberta Agricultural Research Trust was appreciated.

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INTRODUCTION

General

Commercial rapeseed production began in Canada in 1942 as a wartime emergency measure. The oil was required mainly as an industrial lubricant for marine engines. It was also a potential source of an edible vegetable oil. Favorable response in agricultural production stimulated interest in developing a Canadian rapeseed industry to supply domestic requirements for vegetable oil, an alternative crop for agriculture and an export commodity. Today, Canadian rapeseed is a major item in the edible oil markets of the world and Canada has become a major producer-exporter of rapeseed. Among the edible vegetable oils, rapeseed ranks fifth in total world tonnage, being exceeded by soybeans, peanuts, cottonseed and sunflower. Table 1 shows that Canada is, next to India and China, the world's third largest rapeseed producer. China and India produce about two-thirds of the world's rapeseed but in these and most other producing countries domestic consumption exceeds agricultural production. Only about 10% of the world's rapeseed harvest enters into world trade, with Canada, France, Poland, Sweden and Denmark accounting for more than 90% of world exports. In recent years Canada has exported 65-75% of the annual production in the form of unprocessed seed, with Japan, Italy, the Netherlands, West Germany, Belgium and Great Britain being the most consistent importers (Table 2). Recognized as a means of diversifying Canadian agriculture to lessen the dependency

TABLE 1

WORLD RAPESEED PRODUCTION
(1000' Metric Tons)

	1952-56*	1960	1964	1968
Austria	9	6	11	12
Czechoslovakia	33	55	46	73
Denmark	12	13	52	30
France	119	83	247	454
Germany, East	138	182	176	265
Germany, West	33	68	109	170
Poland	100	147	267	712
Sweden	120	61	181	263
U.S.S.R.	38	-	2	4
Canada	41	252	300	540
India	914	1356	915	1568
Japan	276	264	135	68
Pakistan	291	313	302	396
China, Mainland	916	900*	1120*	1070*
Chile	-	41	56	48
Others	110	59	118	60
World Total	3150	3800	4037	5733

*Average of 4 years

Source: FAO Production Yearbook, 1963, 1966, 1969.

TABLE 2

CANADIAN EXPORTS OF RAPESEED
(Tons)

Destination	1962	1966	1970
United Kingdom	1775	4055	6409
Belgium-Luxemburg	2783	8369	8316
West Germany	14783	21710	57323
Italy	90407	60288	35580
Netherlands	31284	40005	100775
Spain	-	28	-
Czechoslovakia	-	-	21813
Pakistan	-	19841	32227
Japan	52309	194498	370732
United States	701	141	161
France	8550	-	12492
Finland	-	-	843
India	-	-	16072

Source: Fats and Oils in Canada/Annual Review, 1967, 1972 (In press). Department of Industry, Trade and Commerce.

on wheat, rapeseed has become a major edible oil seed crop of the prairie provinces (Tables 3 and 4). Alberta and Saskatchewan have about the same acreage under cultivation and together account for approximately 90% of the acreage and 92% of the total crop in Canada. "Rapeseed" encompasses the seed of both spring and winter forms of two species of rapeseed, *Brassica napus* L. ssp. *oleifera* and *Brassica campestris* L. ssp. *oleifera*. Most of the cultivated varieties of rape appear to be variations of one or the other of these two species. Both *B. napus* and *B. campestris* may be grown as annuals or biennials depending upon the variety and upon the sowing season. "Early" varieties, in which the seeds mature in the same year, are known as summer rape while overwintering varieties in which the seeds mature in the second year are known as winter rape. Only annual forms are grown in central Asia and Canada, but in other countries both annual and biennial forms are cultivated. The dominant crop in Europe, Japan and Chile is the biennial or winter form of the *B. napus* species. In Canada, India and Pakistan the spring form of *B. campestris* species is preferred. The species, *Brassica campestris* L., commonly known as summer turnip rape, navette and rübsen (Polish rape) is most extensively grown in western Canada and accounts for approximately 70% of Canadian rapeseed acreage. Polish varieties mature earlier than the Argentine (*Brassica napus*) varieties by about 15 days. This can be crucial in areas where an early frost would stop plant growth at an immature stage. In addition delayed seeding of the turnip rape varieties is frequently used as a wild oat control measure. The seeds of the Polish varieties (Common, Echo, Arlo) are smaller than that of Argentine varieties (Nugget, Oro, Tanka, Target)

TABLE 3

CANADIAN OILSEEDS: ACREAGE, YIELDS, PRODUCTION

	CROP YEAR			
	1965	1967	1969	1971
ACREAGE (Thousand Acres)				
Flaxseed	2320	1023	2341	2011
Rapeseed	1435	1620	2012	5475
Soybeans	265	290	322	360
Sunflowerseed	67	45.8	48	215
Mustard Seed	157	221	267	265
PRODUCTION				
Flaxseed[1]	29.2	9.4	27.6	25.7
Rapeseed[1]	22.6	24.7	33.4	98.5
Soybeans[1]	8.0	8.1	7.7	10.1
Sunflowerseed[2]	29.2	39.6	34.0	152.3
Mustard Seed[2]	127.4	149.9	258	234.8
OIL EQUIVALENT (Millions of Pounds)				
Flaxseed	580	185	545	509
Rapeseed	424	494	668	1970
Soybeans	85	86	81	107
Sunflowerseed	10	15	14	61
Mustard Seed	24	-	-	-

[1] Millions of bushels, [2] Millions of pounds

Source: Fats and Oils in Canada/Annual Review, 1967, 1969, 1972 (In press).

TABLE 4

CANADIAN RAPESEED PRODUCTION

	CROP YEAR				
	1963	1965	1967	1969	1971
ACREAGE (Thousand Acres)					
Manitoba	52	145	145	196	625
Saskatchewan	210	555	600	1000	2750
Alberta	233	735	875	816	2100
Canada (Total)	475	1435	1620	2012	5475
PRODUCTION (Thousands of Bushels)					
Manitoba	860	2400	2300	3500	13000
Saskatchewan	4040	10700	10200	19200	51500
Alberta	3560	9500	12200	14000	34000
Canada (Total	8360	22600	24700	33400	98500

Source: Fats and Oils in Canada/Annual Review, 1967, 1969.

and yields are lower. While seeds of the Argentine varieties are larger and yields superior to the Polish varieties, their longer maturing period renders them susceptible to frost damage. Plant breeding programs to reduce the days to maturity of varieties of _B_. _napus_ to expand the use of these varieties in Canada agriculture are presently in progress.

The steady increase in production of rapeseed in Canada has been accompanied by the development of a domestic industry for oil extraction. Total domestic rapeseed crushing has increased five-fold during the period 1962 - 70 (Table 5). As shown in Table 6, rapeseed oil

TABLE 5

CANADIAN CRUSHINGS OF VEGETABLE OILSEEDS
AND PRODUCTION OF OIL AND MEAL
(Crop Year)
(Millions of Pounds)

	1962-63	1964-65	1966-67	1968-69	1970-71
CRUSHINGS					
Flaxseed	142	162	142	117	158
Soybeans	1072	1172	1193	1203	1406
Rapeseed	81	108	248	347	429
Sunflowerseed	-	23	14	24	32
OIL PRODUCTION					
Flaxseed	49	56	50	41	55
Soybeans	184	201	202	204	241
Rapeseed	31	42	99	141	170
Sunflowerseed	-	7.9	5.6	9.4	12.6
MEAL PRODUCTION					
Flaxseed	86	102	87	72	100
Soybeans	837	930	949	953	1098
Rapeseed	48	63	142	196	249
Sunflowerseed	-	8.6	5.4	9.2	12.0

Source: Fats and Oils in Canada/Annual Review, 1967, 1969, 1972 (In press).

TABLE 6

CANADIAN PRODUCTION OF DEODORIZED FATS AND OILS
(Millions of Pounds)

	1967				1970			
Vegetable Oils	Margarine Oil	Shortening Oil	Salad Oil	Total	Margarine Oil	Shortening Oil	Salad Oil	Total
Coconut	0.8	26.7	-	27.6	0.4	29.1	-	29.5
Corn	X	X	X	24.4	5.8	X	X	25.1
Cottonseed	X	X	X	9.1	1.0	19.9	4.6	25.5
Palm	X	12.5	X	19.9	5.5	16.0	1/	21.5
Palm Kernel	-	9.4	-	9.4	-	9.9	-	9.9
Peanut	X	X	X	26.8	-	X	X	16.1
Rapeseed	32.2	38.7	26.9	101.7	41.4	43.5	45.5	130.3
Soybean	53.9	70.7	29.1	153.7	59.3	98.8	24.9	182.9
Sunflower	X	X	27.6	33.0	1/	5.8	22.1	28.0
Other Vegetable	X	X	-	0.1	1/	0.1	-	0.1
Total Vegetable Oils	106.0	188.7	111.1	405.8	113.5	233.4	122.1	468.9
Total Marine Oils*	32.9	18.8	-	51.7	25.7	12.1	-	37.7
Total Animal Fats**	3.0	72.7	-	75.7	2.8	78.2	-	81.0
TOTAL, ALL FATS & OILS	141.9	280.2	111.1	533.2	141.9	323.6	122.1	587.6

X - Confidential data
1/ - Very small amount
* - herring, seal, whale, other marine oils
** - lard, oleo (all types), tallow (edible)

Source: Fats and Oils in Canada/Annual Review, 1969, 1972 (In press).

utilization has been passing through a phase of rapid growth and is now challenging the dominant role of soybean oil among the vegetable oils, with some margarine brands being made 100% from rapeseed oil. A Federal Task Force on Agriculture (1970) reported that, in addition to export markets, there appears to be considerable opportunity for expansion of domestic consumption of rapeseed oil, and it is conceivable that a doubling of consumption could occur by replacing the vegetable oils presently being imported from other countries.

The rapid development of the rapeseed oil industry has been made possible as a result of extensive research in plant breeding and methods for oil extraction. However, a major by-product of oil extraction, namely rapeseed oil meal, still presents problems in terms of efficient utilization. The amount of RSOM available has increased concurrently with the increased domestic production of rapeseed oil. The meal is high in protein (c. 40%) and as such represents a valuable material for use in animal feed. Utilization as animal feed has been limited by the high crude fibre content (c. 12%) and the presence of various toxic agents derived from glucosinolates. Clearly, as the amount of RSOM available is increasing annually, any process leading to greater utilization of the product would give further economic impetus to the expansion of the rapeseed industry. With this in mind a literature survey was undertaken on the composition, toxicity and detoxification of RSOM. The methods of oil extraction are also considered as the composition of the meal varies according to the extraction process.

Oil Extraction

Processing of rapeseed to obtain oil and meal is similar to that for other high oil content seeds. Recent years have seen the development of new oil extraction procedures resulting in higher quality edible oil and meal. The following processes are, or have been, used in oil extraction of rapeseed:

a) Expeller processing

The first rapeseed extracted in Canada was processed in expeller equipment originally designed for the processing of flaxseed. This is a mechanical process whereby oil is squeezed from the seed. Expeller processing involves crushing, moistening and cooking for approximately 30 minutes at temperatures up to 250^{0}F, followed by oil extraction in a screw press. The primary objective of crushing is a reduction in particle size which assists in uniform cooking and drying. Cooking is conducted to rupture or finish rupturing of oil cells, increase the fluidity of the oil, coagulate the protein aleurone grains which facilitates separation of oil from proteinaceous material, "precipitate" phosphatidic material in order to produce oil of lower refining loss. Moulds and bacteria and enzyme systems are also inactivated. Efficient expeller operation requires that the material be dried to a lower moisture content than required for other extraction methods. Moisture content of rape entering the press will normally be 3% or less. The actual oil extraction of rapeseed is done by forcing crushed, cooked and dried oil-bearing material through the barrel of a screw press or expeller. During this operation, extremely high pressures (15,000 -

20,000 lb/in^2) and temperatures (300^0F) are developed. These high temperatures are detrimental to oil and meal quality. The oil content of the meal ranges from 4 - 7%.

b) Prepress solvent extraction

Preparation of prepress solvent extraction closely resembles that for expeller operation. Cooking and drying conditions are usually less severe, and this results in less heat damage to proteins (Clandinin and Tajcnar, 1961).

Prepressing is similar to standard screw-pressing operations except that it is a relatively mild operation, temperatures and pressures developed during extraction are lower and the material is usually pressed at a higher moisture content. From 70 - 80% of the oil in the seed may be removed. The cake from the expeller, containing 15 - 20% oil, is reground and solvent extracted using normal hexane (b. pt. 60 - 70^0C) in continuous countercurrent extraction apparatus. The solvent is steam stripped from the oilseed cake in desolventizers. The residual oil in meal produced by the prepress solvent extraction method is usually 1% or less.

c) Direct solvent extraction

In direct solvent extraction there is no prepress operation and all the oil is removed by extraction with solvent. This process is usually employed on oilseeds having an oil content of less than 35% (e.g., cottonseed, rice bran, soybeans). Normally high oil content seeds such as rapeseed are not directly solvent extracted, however, a recent process "Filtration-Extraction" (D'Aquin *et al.*, 1953) utilizes

direct solvent extraction of rapeseed. The crushed, cooked seed is forced horizontally in a cylindrical vessel against a counterflow of solvent and oil to effect partial extraction. Finally, the material is deposited on a filtration bed and washed with successively purer batches of solvent until the meal that results contains only 1% oil following desolventization.

Composition of Rapeseed Oil Meal

a) Proximate analysis

The chemical composition of the rapeseed oil and rapeseed meal differ according to the variety and method of oil extraction. The meal, which is the residue remaining after oil extraction, consists primarily of protein and carbohydrate. The proximate analyses of several rapeseed and oilseed meals and feedstuffs are shown in Table 7.

b) Carbohydrate

Matét, Montagne and Buchy (1949) found the carbohydrate content of European rapeseed cake to be similar to linseed cake but slightly higher than that for sunflower cake. Carbohydrate content varied from 20 - 25% while the cellulose content was 8%.

Hrdlicka *et al*. (1964) reported that the saccharide content of fat-free RSOM from *B. napus* was approximately 38%, mainly polysaccharide, with only small amounts of mono-, di-, and tri-saccharides. They reported levels of 0.90% fructose, 1.25% glucose, 1.66% sucrose, 0.35% stachyose and 0.24% raffinose. This is in general agreement with the results of Mizuno (1958) who found: fructose (0.51%), glucose

TABLE 7

PROXIMATE COMPOSITION (Percentage) OF RAPESEED MEAL AND OTHER FEEDSTUFFS

	Dry Matter	Protein	Fat	Crude Fibre	N-free Extract	Ash
Rapeseed Meal						
Expeller						
B. campestris	94.0	35.2	7.0	15.5	29.5	6.8
B. napus	93.2	43.9	6.4	13.7	23.3	5.9
Solvent						
B. campestris	92.0	40.5	1.1	9.3	33.9	7.2
Soybean Meal						
Solvent	89.3	45.8	0.9	5,8	31.0	5.8
Linseed Meal						
Solvent	90.9	35.1	1.9	8.9	39.4	5.8
Sunflower Meal						
Solvent	93.0	46.8	2.9	10.8	24.8	7.7
Fishmeal (Herring)	92.3	70.6	7.5	0.4	3.0	10.8
Meat Meal	93.5	53.4	9.9	2.4	2.6	25.2
Oats	89.1	13.3	5.1	12.0	65.5	4.1
Barley	90.3	12.6	3.0	8.2	62.9	3.6
Wheat	89.1	14.3	1.9	2.9	78.9	2.0

Bowland, J.P., D.R. Clandinin, and L.R. Wetter. 1965.

(0.21%), sucrose (1.11%), raffinose (0.15%) and stachyose (0.19%) and trace amounts of arabinose, galactose, ribose, galacturonic acid, xylose, and rhamnose. In addition, Hrdlicka et al. (1964, 1965) found that the crude fibre, starch and pentosans in rapeseed meal did not change during processing, but the monosaccharide content changed considerably. The more drastic the processing conditions (increased temperature) the greater the losses in mono- and oligosaccharides. The observed decrease in the amount of reducing saccharides as well as decrease in lysine and amino-nitrogen was linked with dark meal color (non-enzymatic browning reaction). Changes in the color of the meal were also related to the thermal decomposition of pigments, such as flavonoids, carotenoids, and chlorophylls at high processing temperatures (115°C). Lodhi (1970) determined the available carbohydrate in rapeseed meal. In a comparative study, using a chick bioassay method (Renner and Elcombe, 1964; Brambila and Hill, 1966), and a chemical method (Clegg, 1956), the author showed rapeseed meal to contain 6.9% and 15% available carbohydrate respectively. Soluble sugars and starch were shown to comprise about 40% of the nitrogen-free extract. The chick bioassay method of determining available carbohydrate would underestimate the carbohydrate if any of the carbohydrate were converted to volatile fatty acids through fermentation in either the crop or caecum. Recent investigations at the Food Research Institute (Ottawa, 1969) have indicated the presence of two types of polysaccharides associated with rapeseed oil meal, the so-called amyloids, which are characterized by their iodine-staining properties and formation of gel-like precipitates on addition of ethanol or glacial acetic acid to their aqueous solution. The rapeseed amyloid

was found to be homogenous and possessed a branched structure with an average unit made up of 25 sugar residues consisting of nine terminal, non-reducing end groups (3 residues of D-galactose, 5 residues of xylose, and one of D-glucose). Siddiqui and Wood (1971) presented a possible structure for the amyloid. The second nonamyloid polysaccharide or the hemicellulose fraction consists primarily of a pure acidic arabinogalactan.

c) Protein

Rapeseed oil meal is a high-protein feedstuff and is a potential substitute for linseed oil meal, soybean oil meal and other plant protein supplements in livestock and poultry rations. The protein content of rapeseed meal has been found to vary depending upon species and environmental conditions. Sosulski and Bakal (1969) extracted and isolated protein from three different varieties of rapeseed, flax and sunflower meals. Varietal differences were noted and the water solubility of the proteins varied markedly. The protein from *B. campestris* seed was less soluble than that from *B. napus* seed. Wetter (1965), reporting the protein content of rapeseed determined by Downey (unpublished data, 1964), stated that de-fatted ground seeds of *B. napus* gave mean values of 47.1 and 48.0% protein (N x 6.25) while the mean values for *B. campestris* were 43.3 and 45.8% protein. In addition, the protein content of meals in the brown soil zones of western Canada were higher than those in the black soil zones. Clandinin and Bayly (1963) also demonstrated varietal differences, that is, *B. napus* being higher in protein content than *B. campestris*. Finlayson *et al*. (1969) demonstrated

species and varietal differences in two proteins previously isolated; these being isolated from eight varieties of rapeseed belonging to *Brassica campestris L.* and *Brassica napus L.* species. These proteins possessed similar chromatographic and electrophoretic characteristics but differed in amino acid composition, particularly with regard to the sulfur-containing amino acids. Sallans (1964) suggested an inverse correlation of protein and oil yield as related to environmental condition; generally, low rainfall and high temperatures are conducive to produce low yields of oilseeds with a high protein to oil ratio. Bunger *et al.* (1936) reported crude protein values for rapeseed meal from 32.8 - 40.9%. Korolczuk and Rutkowski (1971) studied the extractability of nitrogen compounds of de-fatted rapeseed meal as a function of pH and temperature for the isolation of rapeseed proteins. The highest extractability of N-compounds (greater than 80% of total N) was at pH 9.5 - 10 and 30 - 40°C, while the lowest extractability (20 - 25% of the total N) was at pH 6.5 - 8 and 80 - 100°C (region of heat denaturation of proteins) and at pH 3.4 - 4 at 20°C (35% of total N).

The most extensive reports on rapeseed protein are those of Finlayson *et al.* (1969). These researchers extracted the salt soluble proteins from eight varieties of rapeseed using 0.01 M sodium pyrophosphate (pH 7.0) and 1.0 M sodium chloride and separated them into a number of components. The pyrophosphate extract contained two major proteins, a neutral 12S protein and a basic 1.7S protein. Together, they constituted 30% of the nitrogen in the extract. The 1.0 M sodium chloride also contained a 12S protein which accounted for 21% of the nitrogen in the extract and resembled the 12S component of the

pyrophosphate extract. Both proteins (12S and 1.7S) from different varieties had similar electrophorectic and chromatographic behavior but differed in amino acid composition, especially sulfur-containing amino acids.

The quality of the protein, or amino acid composition, in rapeseed oil meal has changed with improved processing technology. Approximately 72% of rapeseed meal nitrogen occurs in the amino compounds; N-amide accounts for 12%, while non-soluble nitrogen and products not precisely identifiable, such as those of thiocyanate degradation, constitute 16% (Andre and Delaveau, 1954). Initially, rapeseed was processed in plants originally designed for the extraction of linseed, consequently, most of the meal was produced by the expeller method; today, meals are produced by either the prepress-solvent or by the solvent process (Youngs, 1965). Modern meals are subjected to less heat during processing and the amount of oil left in the meal has been reduced as compared to expeller meals. Protein denaturation has been observed in the processing of several oilseeds including soybeans (Evans and Butts, 1948); sunflower (Renner _et al._, 1953); cottonseed (Conkerton _et al._, 1957); peanuts (Bensabat and Frampton, 1958); mustard seed (McGhee _et al._, 1964); sesame (Carter _et al._, 1961) and rapeseed (Clandinin _et al._, 1959), (Clandinin and Tajcnar, 1961). The basic amino acids, lysine, arginine, and histidine, as well as cystine and tryptophan have been reported to be affected. Lysine appears to be the most heat sensitive. Several workers have indicated that rapeseed is an inferior meal because of its low lysine content (Kratzer _et al._, 1954; Klain _et al._, 1956; Blaizot and Poliakoff, 1959). Clandinin _et al._ (1959) noted that meals processed at high temperatures were nutritionally inferior and low in lysine. Protein analyses of two expeller-processed

commercial meals were 43.3% for *B. napus* and 33.9% for *B. campestris*. Manns and Bowland (1963) found the protein content of two solvent-processed meals of *B. campestris* to be 36.7% and 34.7%. Two types of heat damage occur. In one, the amino acids are bound in such a form that they are not liberated by digestion *in vivo* or by enzyme hydrolysis *in vitro*, but are liberated by acid hydrolysis. In the second case, the amino acids appear to be irreversibly lost and may not be recovered on acid hydrolysis (McGhee, 1964; Evans and Butts, 1948). Ballester *et al*. (1970) revealed essential amino acid pattern of rapeseed meal resembling the protein quality of casein, that is, methionine being the limiting amino acid. These results substantiated the findings of Bowland, Clandinin and Wetter (1965).

Clandinin and Tajcnar (1961) established a correlation between lysine content and processing temperature of expeller-processed rapeseed meals. They also noted a direct relationship between lysine content and the final oil content of the processed meal; generally, a marked reduction in lysine was associated with oil content (expeller extraction) less than 6%. In any event, it is evident that the essential amino acid composition of modern rapeseed meals compare quite favorably with soybean and other oilseed meals (Table 8). Varied opinions exist concerning amino acid content and rapeseed varieties. Clandinin and Bayly (1963) determined and compared the amino acid content of six different varieties of rapeseed grown in Alberta. Differences in protein content and amino acid distribution in the protein of the seed attributable to varietal or strain effects were noted. Significant differences in lysine and histidine

TABLE 8

AMINO ACID CONTENT OF VARIOUS RAPESEED MEALS AND OTHER PROTEIN SUPPLEMENTS
(g amino acid/16 g nitrogen)

		Rapeseed[1]									
Amino Acid	Soybean[1]	Expeller	Solvent	Sunflower[1]	Fish meal[1]	Cottonseed[2]	Peanut[2]	Linseed[2]	Safflower[2]	Sesame[2]	Castor[2]
Alanine	4.5	4.21	4.43	5.1							
Aspartic acid	10.8	6.58	6.94	9.1							
Arginine	8.3	5.90	5.52	9.1	5.9	11.0	10.3	9.3	7.8	11.9	10.0
Cystine	1.7			1.8	1.0						
Glutamic acid	18.0	16.16	17.50	18.8	0						
Glycine	4.4	4.68	4.97	5.6							
Histidine	3.3	2.40	2.76	2.8	2.4	2.7	2.2	1.8	2.0	2.2	1.7
Isoleucine	5.0	3.71	3.70	4.2	4.0	4.0	4.3	4.6	3.8	4.3	4.6
Leucine	8.1	6.45	6.87	6.9	10.0	6.2	6.7	6.0	5.5	6.9	5.6
Lysine	6.5	4.39	5.60	3.5	5.7	4.2	3.5	3.6	2.7	2.8	3.0
Methionine	1.8	1.88	1.95	2.2	3.0	1.5	1.0	1.7	1.5	2.6	1.5
Phenylalanine	4.8	3.74	3.94	5.1	4.8	5.2	5.0	4.5	5.2	4.7	4.7
Proline	5.5	5.71	6.50	4.5							
Serine	5.8	4.03	4.35	4.6							
Threonine	3.7	4.08	4.36	3.4	5.0	3.5	3.0	3.8	2.9	3.6	3.2
Tryptophan	1.5	0.94	1.28	1.4	1.2	1.6	1.2	1.7	1.2	1.9	1.1
Tyrosine	3.8	2.16	2.18	2.9	2.8						
Valine	5.1	4.76	4.89	5.8	4.0	5.0	4.8	5.6	4.9	5.1	5.4

[1] Wetter, L.R. 1965

[2] Lyman _et al_. 1956

content were observed; however, no significant varietal differences with respect to the other essential amino acids studied were found. The Argentine varieties were significantly lower in lysine than the Polish variety. In addition, environmental effects on the protein quality of rapeseed as measured by the essential amino acid content seemed to be primarily associated with lysine. This data is contrary to the findings of Miller *et al*. (1962) who, in a detailed study of the amino acid composition of the seed meals of forty-one Cruciferae species, compared *B. napus* and *B. campestris* and found little difference in amino acid content. Tristram and Smith (1963) and Finlayson (1965) suggested amino acid content variability as a result of the lack of standardization of amino acid analysis procedures.

d) *Lipid*

The processing of rapeseed to obtain oil and meal is similar to that for any other high oil content seed, such as corn or linseed. One obvious effect of the method of processing upon the quality of the meal is the quantity of residual oil remaining in the meal after extraction. Little has been reported on the lipid composition of rapeseed meals and lipids may well be an important factor in the acceptability and stability of the meal. In processes involving solvent extraction, the residual fat in the meal is reduced to 1 - 4% whereas in conventional expeller-pressed meals, this may vary from 6 - 7% (Clandinin *et al*., 1959; Mustakas *et al*., 1965; Ballester *et al*., 1970a). Zeman *et al*. (1964) observed similarities in fatty acid composition of rapeseed meal and rapeseed oil, providing the residual content exceeded 1%.

Changes in the lipid composition of the meal are greater the more exhaustive the extent of extraction. The composition of fatty acids in the residual oil in rapeseed meals revealed a higher content of palmitic, oleic and linoleic acids than in the average composition of fatty acids in rapeseed oil, but less erucic, eicosanoic and linolenic acids. Typical analysis of rapeseed oil is illustrated in Table 9.

TABLE 9

FATTY ACID COMPOSITION OF CANADIAN RAPESEED OILS

	Fatty Acids, Percent						
Species and Variety	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{22:1}$
B. napus							
Golden	3.3	1.1	18.6	14.0	7.4	13.4	41.8
Nugget	3.3	1.5	22.8	12.2	5.4	14.2	40.6
Zero erucic	4.7	1.8	63.3	20.2	8.9	1.3	0.0
B. campestris							
Arlo	3.2	1.1	26.6	17.5	8.8	11.8	31.0
Yellow Sarson	1.8	0.8	11.7	10.5	8.3	5.9	61.0
Zero erucic	4.3	0.1	54.8	31.1	9.7	0.0	0.0

Downey, R.K. 1963.

Varietal and environmental changes in fatty acid composition of rapeseed has been reported by numerous researchers (Eckey, 1954; Craig and Wetter, 1959; Craig, 1961; Stefansson *et al*., 1961; Harvey and Downey, 1964;

Downey, 1966; Dembinski *et al.*, 1967; Appelqvist, 1968). McKillican and Lacrose (1970) investigated residual lipids of rapeseed meals (*B. campestris*) produced by pilot-scale and commercial-solvent extraction processes. The "free" lipid class distribution of residual lipids was similar to that of the hexane extract of intact mature seed, almost enitrely triglyceride. The amount of residual "free" lipid varied with the method of meal production. A large number of components were found in the "bound" residual lipids than were previously reported in the polar lipids of the hexane extract. There were differences in the glycolipids; more sterol glycoside and esterified sterol glycoside were found in the residual lipids, whereas more mono- and di-galactosyl diglyceride had been found in the polar lipids of mature seeds. The polar lipids of the hexane extract of mature seed and "bound" residual lipid of the meals had similar phopholipid fractions. In both, phosphatidyl choline was the main component, whereas phosphatidyl ethanolamine was also present. Sallans (1964) observed a direct correlation between oil content (fatty acid composition) and environmental conditions such as rainfall, soil type and fertilizer practices.

e) Crude fibre

Lignin and insoluble carbohydrate material (cellulose) compose the crude fibre segment of animal feeds. Rapeseed oil meal has a higher fibre content than other oil meals. The values generally range from 9 - 16% and the fibre content is not different when solvent or expeller-processed meals are compared (Klain *et al.*, 1956). Rapeseed oil meal has a higher fibre content than soybean meal (2.5 times) but it is only

slightly higher than sunflower or linseed meal. Ballester _et al_. (1970) investigated samples of commercially-extracted rapeseed presscake meal (_B. napus_). Crude fibre content of the meals varied from 13.2 - 14.7%. Research conducted by Youngs (NRC Saskatoon, 1967) illustrated that dehulling of rapeseed seed coat prior to oil extraction has the desirable effect of reducing the fibre content and increasing the protein content of the meal. The seed coat or hull is low in oil and protein and high in polysaccharides. However, the hull contains 6 - 7% of both the oil and protein in rapeseed and it is doubtful if the increased value of the meal could offset such a loss unless the hull could be sold as a feed constituent at approximately one-half the price of the non-dehulled meal.

f) Ash and vitamins

The ash content, which indirectly is a measure of the mineral content of the rapeseed oil meal, varies depending on the source of the seed. The ash content for meals obtained in western Canada vary from 6 - 7% (Klain _et al_., 1956), a value lower than found in Sweden. Published data reveals that rapeseed meal is relatively rich in calcium and phosphorus. Burkitt _et al_. (1954) reported calcium contents of 0.51 and 0.61% and phosphorus contents of 0.80 and 0.84% in rapeseed oil meal. The calcium and phosphorus content of Canadian meal is 0.6 and 1.1% respectively compared with 0.3 and 0.7% for typical soybean meal and 0.4 and 0.8% for linseed meal (Bell _et al_., 1967). Karvanek _et al_. (1964) reported that 40% of the phosphorus content of rapeseed meal appear in the form of phytin compounds. The occurrence of phytin phosphorus in rapeseed meal was also studied by Becker (1950). Sawhney

and Kehar (1961) reported the manganese content of rapeseed meal to be 153.5 ppm., considerably higher than other vegetable seed meals (39.5 - 80.0 ppm.). Values of 1.9% total phosphorus, 0.8 phytin phosphorus, 0.94 mg copper/100 gm, 30.05 mg iron/100 gm, 0.26 mg nickel and 7.52 mg manganese/100 gm *B. napus* meal was reported by Hrdlicka *et al.* (1964).

In rapeseed meals as in other oil seed meals, the majority of vitamins appear to be members of the B-group. Klain *et al.* (1956) reported no significant difference in vitamin content of two varieties of rapeseed. In comparison with meals from other vegetable sources rapeseed oil meal appears to be richer in choline and niacin, similar in riboflavin and lower in thiamine and pantothenic acid. The tocopherol content of rapeseed meal is about 45 mg/kg, including 12 mg of the alpha form (Ames, 1956; Hjarde *et al.*, 1962). The mineral and vitamin content of several oilseed meals is shown in Table 10.

Toxicity of Rapeseed Oil Meal

The presence of toxic components (goitrogens) is a yet unsolved problem in the utilization of rapeseed meal. The existence and evidence of goitrogenicity of rapeseed oil meal has been known for many years. The first report on the goitrogenic properties attributed to components of rapeseed appeared in 1941. Kennedy and Purves (1941) showed that *Brassica* seeds produce thyroid hyperplasia when fed to rats. Petit *et al.* (1944) reported that, while 14% rapeseed oil meal in chick starters was satisfactory, considerable mortality resulted when the ration was increased to 20%. Numerous other reports (Turner, 1948; Blakely and Anderson, 1948; Allen and Dow, 1952) have confirmed thyroid

TABLE 10

MINERAL AND VITAMIN CONTENT OF OILSEED MEALS

	Rapeseed meal[1, 2]		Peanut meal[3]	Cottonseed meal[3]
Vitamin (mg/kg)	*B. napus*	*B. campestris*		
Choline	7000	6450	1800-2400	3200-3500
Pantothenic Acid	9.9	8.6	15-53	7-14
Thiamine	1.9	1.7	1.6-17.2	3.9-14.2
Riboflavin	4.2	3.3	2.0-5.3	5.3-9.0
Niacin	167.0	152.0		38.6-45.0
Minerals (p.p.m.)				
Ca	3000-6000		70-200	1800-3300
P	9000-19000		3500-5700	11000-12600
Na	400		50-700	200-2000
K	151000		11000-12000	12000-14600
Mg			22000-5100	5400-5900
Mn	75		18-550	23-280
Fe	300		30-100	80-970
Cu	10			17-19

TABLE 10 -- Continued

Sunflower meal[3]	Soybean meal[3,4]	Sesame meal[3]	Safflower meal[3]	Linseed meal[3]
4290	27.40	1496	3250	1210-1848
33-58	14.5	902	39.6	14.1-17.6
3.6	6.6			
3.6	3.3	4.62	2.42	3.86-3.52
90-415	26.8	110.88	22.0	28.6-35.2
3600-4900	2600	21200-24600	4000	4000
8400-10500	6200	13800-14200	13000	8500-9000
	1400			
	20600	13500		11000-14000
	3000	7800		
		38-50		
	140			

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[1] Klain et al. 1956

[2] Defremont, C. 1964

[3] Altschul, A.M. 1958

[4] Nat. Acad. Sci., Nat. Res. Council, Washington, D.C. 1956 Pub. 449

enlargement associated with feeding rapeseed oil meal to poultry. Clandinin _et_ _al_. (1959) has recently reported deleterious effects from the use of rapeseed oil meal in chick rations, with Argentine type rape being more toxic than Polish type. Bell (1957) observed thyroid enlargement due to feeding of Argentine rapeseed oil meal to mice. Numerous workers (Norfeldt _et_ _al_., 1954; Fevrier, 1957; Hussar and Bowland, 1959; Manns _et_ _al_., 1963; Manns and Bowland, 1963) have reported goitrogenic effects as a result of rapeseed oil meal feeding to swine. It has been observed that the rat and pig generally respond very similarly to rapeseed oil meal feeding at various physiological stages in the life cycle. Ruminant animals do not seem to be affected by the potential goitrogenic factors in rapeseed oil meal to the same extent as poultry, swine and laboratory animals (Bell, 1955; Hornoiu and Cadantu, 1960; Bezeau _et_ _al_., 1960; Asplund, 1961; Virtanen _et_ _al_., 1963).

Goitrogenic substances are not restricted to rapeseed, nor, for that matter, to the _Brassica_ family, nor does the evidence indicate that the same factor is involved in all members of the _Brassica_ family. The undesirable components appear in the seed in the form of glucosinolates, also called thioglucosides, which are biologically inactive. The destruction of the cellular structure allow the glucosinolates to come in contact with an endogenous seed enzyme. This enzyme, called myrosinase or thioglucosidase glucohydrolase enzyme (EC 3.2.3.1) hydrolizes the glucosinolates liberating glucose, bisulphate and certain biologically active components termed "mustard oils" (isothiocyanates and oxazolidinethione). Isothiocyanates carrying a β-OH-group cyclize spontaneously to oxazlidinethiones. Other hydrolysis products, for

example nitriles, have also been isolated (Van Etten *et al*., 1966). Gadamer (1897) and Ettlinger and Lunden (1957) have proposed reaction mechanisms of thioglucoside cleavage by myrosinase. Ettlinger and Lunden (1957) proposed a mechanism of myrosinase action upon the thioglucoside segment of rapeseed (Fig. 1). Some controversy has existed in earlier years whether myrosinase is a one- or a two-enzyme system, that is, a thioglucosidase capable of splitting the glucose moiety and a sulfatase capable of removing sulphur. Gaines and Goering (1962) conclusively established the dual nature of myrosinase. Greer (1962) obtained results of enzymes in the gastro-intestinal tract capable of thioglucoside cleavage. In addition, *Escherichia coli* and *Aerobacter aerogenes* were found to possess appropriate enzyme systems.

In 1901, Sjollema identified crotonyl isothiocyanate as a constituent of the essential oil fraction of rapeseed. Viehover *et al*. (1920) working on methods for identifying rapeseed importations, found crotonyl- and allyl-isothiocyanates to be present in rape and mustard seed respectively, and established their relative toxicities. Andre and Delaveau (1930) found evidence in rape for the presence of three individual isothiocyanates but were unable to suggest their chemical nature. Kjaer *et al*. (1953, 1956) confirmed the presence of three volatile mustard oils. The volatile isothiocyanates in rapeseed were identified as two major ones, 3-butenyl isothiocyanate (CH_2=$CHCH_2CH_2NCS$) and 4-pentenyl isothiocyanate (CH_2=$CHCH_2CH_2CH_2NCS$) and one minor one 2-phenylethyl isothiocyanate. There are some differences in the proportions of the two major isothiocyanates; in *B. napus* the predominant one is 3-butenyl isothiocyanate (Ettlinger and Hodgkins, 1954; Kjaer *et*

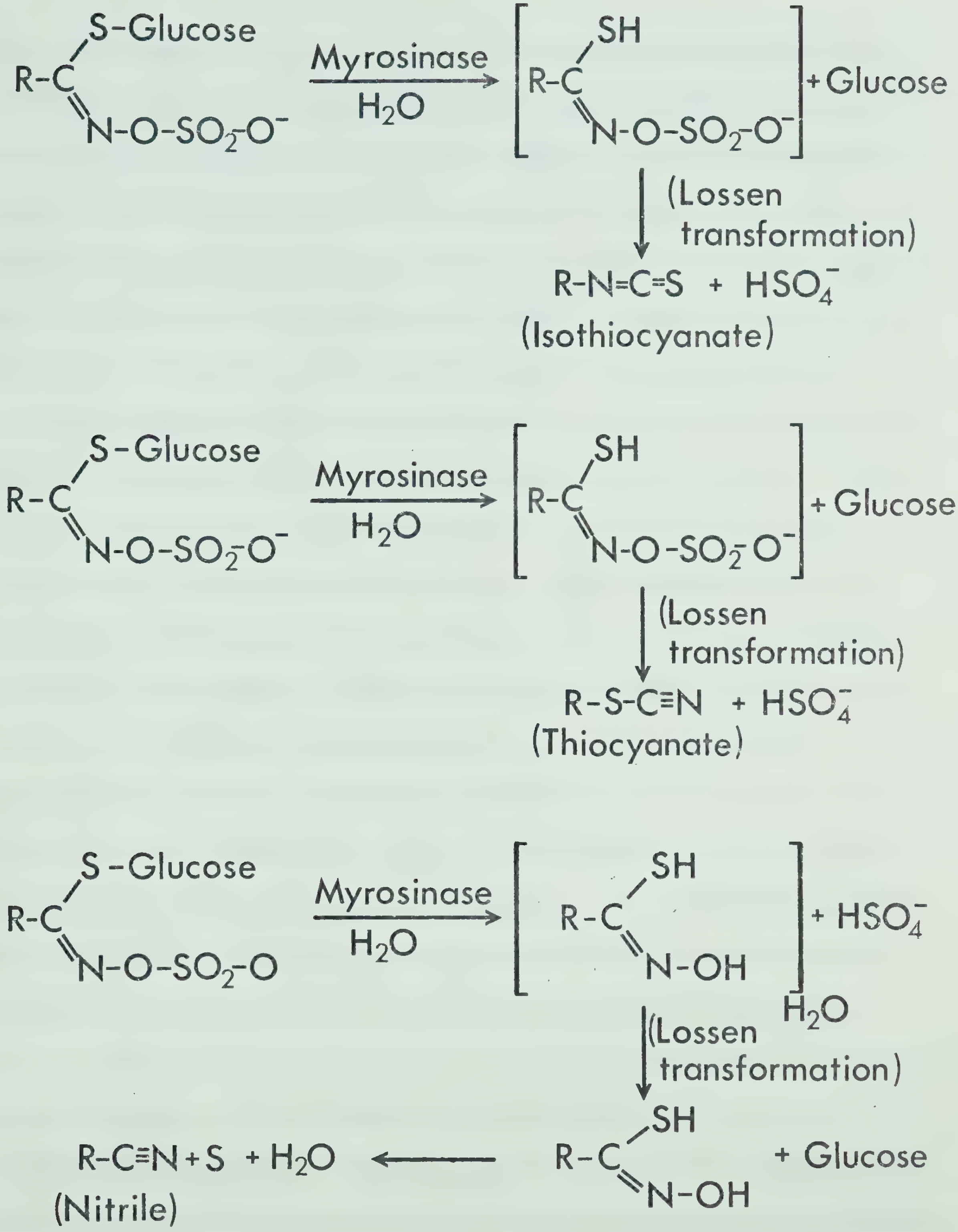

Fig. 1. Reaction Mechanism of Myrosinase on Thioglucosides (Ettlinger and Lunden, 1957).

al., 1953) while in *B. campestris* the two major isothiocyanates are present in approximately equal proportions (Youngs, 1964). Josefsson and Appelqvist (1968) pointed out that these anti-nutritional factors occupy a significant proportion of the fat-free substance: typically about 3% in *Brassica campestris*, 4-5% in the summer types of *B. napus* and slightly more, 6-7% in the winter types. In 1949, Astwood *et al*. and Carroll isolated a goitrogenic compound from *Brassica* seeds, including rapeseed, that was later proven to be L-5-vinyl-2-thiooxazolidone. This compound was given the descriptive name: goitrin. These workers also found that goitrin exists in the seed as a glucoside (progoitrin). Ettlinger (1950) and Greer (1962) isolated these two components. Matét *et al*.(1949) isolated the glucosides sinigrin and gluconapin from rapeseed; these substances were shown to be precursors to allyl- and crotonyl-isothiocyanates. Astwood *et al*. (1949) established a possible relationship between the isothiocyanates and thiooxazolidone. Raciszewski *et al*. (1955) isolated thiooxazolidine from rapeseed and later synthesized the substance. Bell and Belzile (1965) stated that the only isothiocyanates having significant antithyroid activity are those capable of cyclizing to form oxazolidinethione.

There exists variation in the "mustard oil" content of rapeseed grown in western Canada. Wetter and Craig (1959) found that the isothiocyanate content of Argentine and Polish types of rapeseed varied from 4.33 - 5.36 mg per gm of oil-free meal, while the oxazolidinethione content varied from 1.33 - 5.60 mg per gm of meal. The isothiocyanate content was similar for both types but the oxazolidinethione content was approximately 3.5 times higher in the Argentine type of rapeseed

(Argentine, Swedish, Golden and Regina II). Similarly, Clandinin *et al.* (1959) showed that *B. campestris* had a lower oxazolidinethione content than *B. napus*. Daxenbichler *et al.* (1964), in a study of the "mustard oil" content of seed meals from 65 species of Cruciferae reported values of 5.9 - 6.0 and 4.3 - 6.2 mg per gm for isothiocyanate and oxazolidinethione respectively in *B. napus*. In the earlier expeller meals where water was added during cooking and high temperatures were reached in the expeller, some hydrolysis of the thioglucosides would be expected. Wetter and McConnel (Bell, 1955) found isothiocyanate contents (expressed at allyl isothiocyanate) varying from 0.3 - 1.4% in solvent-extracted rapeseed oil meals. Samples of Polish, Argentine and Turkish types were tested and the highest values were found in the Turkish samples. Clandinin (1964, unpublished data) reported isothiocyanate and oxazolidinethione values of 2.44 and 2.40 gm/kg respectively in expeller-processed meals and values of 4.18 and 3.58 gm/kg respectively in prepress plus solvent-extracted meals. Ballester *et al.* (1970) in a study of samples of solvent-extracted rapeseed presscake meal (*B. napus*) from local Chilean industries reported isothiocyanate and oxazolidinethione content in agreement with values reported by Youngs and Wetter (1967), Appelqvist (1962) and Wetter and Craig (1959).

Environmental differences and "mustard oil" content have been investigated by numerous workers. Clandinin *et al.* (1959) indicated that environmental parameters affect the oxazolidinethione content to a greater degree than isothiocyanate content. Studies of glucosinolate content in seed of rape and turnip rape as affected by variety and environment were made by Josefsson and Appelqvist (1968). Seed meals

of winter and summer types of varieties of *B. napus* and *B. campestris* illustrated considerable differences in amounts of glucosinolates between species and between summer and winter types of the same species. Varietal differences were relatively small in most of the material studied. By fertilizer experiments under laboratory conditions, they showed that low application of sulphate fertilizer resulted in a low content of glucosinolates in the seed. No significant variation in glucosinolate content was found between different levels of nitrogen fertilizer. In contrast, Trzebny (1964) found, however, in a study of field-grown material that a high level of nitrogen fertilizer decreased the glucosinolate content and increased the protein content whereas a direct correlation between glucosinolate content and sulphate fertilizer was established.

The main sources of thiocyanates are glucobrasicine and sinalbine which are present in rape and turnip-rape seeds. The isothiocyanates obtained from these compounds can be transformed into thiocyanates (Ettlinger and Lundeen, 1957). The isothiocyanates have a considerably lower goitrogenic activity than the oxazolidinethiones; the thiocyanates are characterized by the weaker goitrogenic activity (Fertman and Curtis, 1951; Gmelin and Virtanen, 1960). The influence of isothiocyanates and thiocyanates are similar; they block the capture of iodine by the thyroid gland and set free the iodine accumulated in it. In this way they check the first phase of the thyroid hormones biosynthesis, but they do not damage any of the thyroid gland mechanisms. The goitrogenic effect of these compounds can be removed by the addition of iodine to the ration. Goitrin (L-5-vinyl-2-oxazolidinethione) and

nitriles are formed from the glucosinates, progoitrin and epi-progoitrin. Under the influence of myrosinase cyclic isothiocyanate or L-5-vinyl-2-oxazolidinethione is formed, a compound ranking high in antithyroidal activity. The mechanism of goitrin influence on the thyroid gland is not completely understood, however, it is known that it blocks the irreversible mechanism connected with the organic binding of iodine in the thyroid, and thus a partial suppression of tiroksine synthesis follows. The decrease of tiroksine in the blood circulation resulting from this partial suppression, stimulates the hypophysis to secrete excessive amounts of thyrotropine affecting the thyroid gland, stimulating its growth (Clandinin and Tajcnar, 1961). The goiter caused by addition of these compounds to the diet cannot be alleviated by increasing the iodine intake; thus damage can occur when meal containing these compounds is used in the feeding of nonruminants. Recent investigations (Langer, 1966) have demonstrated a synergistic effect of the goitrogenic components (oxazolidinethione, allyl isothiocyanate, thiocyanates) of rapeseed oil meal. These investigations were conducted on rats receiving the goitrogens individually or as mixtures in their diets. Rapeseed meals have been used in cattle feeds in Europe for many years with no ill effects. The major disadvantage in ruminant nutrition being not of toxological significance, but rather a matter of palatability (presence of a bittering substance, sinapin) and digestibility (higher crude fibre content).

Detoxification of Rapeseed Oil Meal

The ultimate solution to the problem of toxic components in RSOM would be to breed plant varieties free from glucosinolates. Considerable progress had been made in this direction (Appelqvist, 1962; Krzymanski, 1970). Downey *et al.* (1967) reported plant success of plant breeding and removal of glucosinolates, thereby increasing meal quality. Within the *B. napus* species, plants of the variety Bronowski were found to have extremely low levels of all three major compounds (3-butenyl and 4-pentenyl isothiocyanates and L-5-vinyl-2-oxazolidinethione). In the *B. campestris* species, selection within the varieties Echo and Arlo, adapted to western Canada, resulted in isolation of plants with seeds free of the thioglucosides, giving rise to 4-pentenyl isothiocyanate, oxazolidinethione or both. Genetic investigations on these two compounds in *B. campestris* showed that they are independently inherited and that their absence is recessive in character. Results suggest that complete removal of all thioglucosides from both rapeseed species may be possible.

Another approach to the problem has been the development of methods to detoxify the meal. Many techniques have been investigated with varying degrees of success. Bell (1957) conducted feeding and growth experiments with mice receiving diets containing rapeseed oil meal treated by various steam processings, by extractions with hot or cold water, by extractions with ether or alcohol, by partial or complete sterilizations and by acid hydrolysis. The conclusions drawn from this study indicated that steam treatments of up to 20 minutes at 15 pounds pressure did not counteract the toxic factor. Hot water extraction of the meal was only partially effective. Similar results were reported

with hot alcohol extraction. Despite the improvement in nutritional quality, the extraction procedures did not improve the rapeseed oil meal sufficiently to equal the control ration of soybean oil meal and meat meal. Acid hydrolysis (concentrated hydrochloric acid at 100°C for 10 min) resulted in little improvement in rapeseed oil meal toxicity. Attempts have also been made to hydrolyze the glucosinolates at elevated temperatures with weak solutions of sulphuric acid, but apart from removing the glucosinolinates, this method caused considerable destruction of lysine (Szewczuk et al., 1969).

Considerable amounts of goitrogenic substances can be removed through the application of organic solvents. Matet et al. (1949) reported removal of toxic glucosides by extraction with 70% aqueous ethanol or by fermentation and subsequent solvent extraction and steam distillation. Allen and Dow (1952) demonstrated a marked inhibition of goitrogenic activity (determined by chick feeding experimentation) could be obtained either by hot water extraction of the meal (98 - 99°C) or by the addition of iodine to poultry diets in the form of inorganic iodide. Grussendorf (1953) reported that hot water extraction of rapeseed oil meal was effective in removing the toxic principle and that RSOM had been used successfully in Europe as an ingredient of silage. Van Etten et al. (1965) demonstrated glucosinolate extraction with 75 - 80% methanol. Reduced losses of proteinaceous material at pH 3.5 - 4.5 or 8 - 9 was observed. The same authors extracted glucosinolates from defatted *Crambe abyssinica* meal with 75% acetone - 25% water, but it resulted in a loss of more than 20% of the meal solids. Thioglucoside extraction with acetone or its mixture with hexane has also been reported (Tookey et al.

1965). In 1968, Shaikh et al. in Pakistan, prepared a glucosinolate-free protein from commercial rapeseed cake and studied its properties. Defatted meal was extracted with aqueous NaOH (pH 8 - 11) and precipitated at pH 4.5 or 6; pH 10 for solution and pH 4 for precipitation proved most satisfactory. This isolate was shown to be free of isothiocyanates and nutritionally superior to that of the original meal. The Net Protein Utilization of the isolate was 69%, that of the original meal, 53% and those of 50:50 mixtures of isolate plus fish flour and skim milk powder 99% and 88% respectively. Ballester et al. (1970) studied rapeseed meal and its detoxification. Aqueous extraction at room temperature reduced the content of both isothiocyanates and oxazolidinethione but a "double water extraction" (one extraction lasting from 8 - 14 hours followed by a second of 1 - 3 hours duration) was considered to be the most satisfactory method. The latter process reduced oxazolidinethione and isothiocyanate content of rapeseed oil meal 84 and 77% respectively. These authors reported increased net palatability and protein levels corresponding favorably to those of animal proteins, from 40 - 69%.

Dry heating of rapeseed oil meal at 150°C destroys or inactivates the toxic principle, as claimed by Frolich (1953). Belzile et al. (1963) subjected solvent-extracted meals to twelve hours dry-heat treatment at 135°C. Such treatment destroyed myrosinase activity but enzyme inactivation was extremely slow, nevertheless, dry-heated meals were superior to unheated meals when fed to mice in bioassay experiments. The authors also illustrated that steam treatment (autoclaving) had a marked effect upon the rate of disappearance of isothiocyanates and oxazolidinethione. At pressures of 17 lb/in^2 for periods up to two

hours over 90% of the original oxazolidinethione and 75% of the isothiocyanates had disappeared. Destruction of toxic factors by extended steam treatment under pressure was confirmed by animal tests. Rutkowski *et al.* (1966) found that calcination of RSOM at 120°C destroyed 80% of the oxazolidinethione. Only traces of goitrogenic substances remained after calcination at 130°C. The dry-heat treatment to destroy the myrosinase and thereby to avoid the formation of toxic principles in the meal is at present used in rapeseed processing plants. In present commercial practice crushed rapeseed is cooked for 30 minutes without the addition of water at a temperature of 176 - 194°F to inactivate the myrosinase (Youngs, 1965). This operation leaves the thioglucosides intact in the meal. However, if RSOM is used for food purposes, the toxic principles may be liberated by certain bacteria, especially *E. coli* and *A. aerogenes* present in the gastro-intestinal tract (Greer, 1962). Goering *et al.* (1961) reported partial detoxification of expeller- and solvent-extracted rapeseed meal by an enzyme treatment (sinigrinase). Free isothiocyanates and glucosidic compounds were removed from RSOM following enzyme digestion by steam distillation. Belzile *et al.* (1963) formulated a steam stripping procedure whereby isothiocyanate removal was complete within two hours and about 10% of the oxazolidinethione remained after three hours. However, these workers observed gradual deterioration in protein quality (lysine destruction) as time of the steam treatment was extended. An early example of this technique, steam distillation, was used by André (1955) in an attempt to detoxify and remove the pungent flavor from rapeseed oil meal.

A wet heat treatment for myrosinase destruction and improved oil quality was developed at the Food Research Institute (FRI) of the

Research Branch, Canada Department of Agriculture (Eapen et al., 1968); more recently, Eapen et al. (1969) showed that a white, bland, defatted, thioglucoside-free flour could be prepared from rapeseed. The thioglucosides were removed by aqueous extraction at ambient temperature (near 20^{o}C). Wet heat treatment of the meal prior to water extraction inactivated myrosinase and it also facilitated the removal of seed coat resulting in a more attractive meal. The seed coat fraction, also free of thioglucosides, was 33% protein and could be utilized as animal feed. Rat-feeding tests (Tape et al., 1970) were conducted to determine PER (Protein Efficiency Ratio) using casein as an internal standard. The PER value of the hull-free rapeseed flour was the same magnitude as casein, whereas hull-rich rapeseed meal was below casein.

Alternatives to enzymatic degradation of thioglucoside have also been studied. Austin et al. (1968) acknowledged nonenzymatic degradation of crambe seed meal thioglucoside (epi-progoitrin) by ferrous salts. The reaction products formed by the action of ferrous salts on epi-progoitrous were a nitrile, (S)-1-cyano-2-hydroxy-3-butene, and a thionamide, (S)-3-hydroxy-pent-4-enethionamide. Kirk et al. (1971) investigated decomposition of thioglucosides in Crambe abyssinica meals with chemical additives. Salts of iron and copper, especially ferrous sulphate, were preferred because they did not reduce the lysine content as did the alkalies. All additives were effective in decomposition of epi-goitrin. Rats fed ferrous sulphate treated crambe meal as 30% of a protein sufficient diet gained 70% compared with a basal control, however, enlargement of thyroid, liver and kidneys of test animals was observed. Heywang (1957) and Cavanagh (1960) added ferrous sulphate to cottonseed

meal without apparent toxic effects. An ammoniation process for thioglucoside destruction in crambe seed meal was demonstrated by Kirk _et al_. (1965). Feeding experiments with chicks and cattle illustrated improved palatability and nutritional quality of the meal. Mustakas _et al_. (1966) developed a soda ash (sodium carbonate) process that deactivates unpalatable and growth-inhibitory factors (thioglucosides) in untreated crambe meal. This treated meal was used commercially in 1965. Similarly, animal feeding experiments confirmed improved palatability and nutritional quality of the meal.

The National Research Council of Canada (Youngs, 1967a) announced the development of a new chemical treatment of rapeseed meal (ferrous sulphate) designed to destroy potentially goitrogenic compounds. The process consisted of the treatment of rapeseed meal with an aqueous solution of ferrous sulphate and subsequent steam-stripping. Ferrous sulphate decomposed the thioglucosides to give organic nitriles rather than the isothiocyanates and oxazolidinethione formed on enzymatic hydrolysis. Some of the nitriles formed were steam volatile, such as 1-cyano-3-butene and 1-cyano-4-pentene and readily steam-stripped from the meal. However, of the decomposition products, a hydroxynitrile (1-cyano-2-hydroxy-3-butene) corresponding to the oxazolidinethione was found to be only slightly steam volatile and remained in the meal. The Polish varieties, _B_. _campestris_, gave only very small quantities of the hydroxynitrile, while _B_. _napus_ produced larger quantities. This hydroxynitrile was shown to be toxic to animals if incorporated into the diet at too high a level; consequently, the process was limited to meals low in thioglucoside. The "progoitrin" content of the meals to be

processed by this method should not exceed 2%, corresponding to a hydroxynitrile content of 0.5% by weight. Meals successfully detoxificated by the procedure were summer turnip rape (*Brassica campestris* var. *oleifer* f. *annua* commonly called Polish type rapeseed) and Brown or Oriental Mustard, *Brassica juncia*. According to Kirk *et al*. (1971) ferrous-treated rapeseed meals had been successfully incorporated in the diets of mice and swine. Youngs *et al*. (1971) were granted a patent for the use of ferrous sulphate in the catalytic decomposition of progoitrin in rapeseed meal.

A most interesting new method of biological detoxification was reported by Staron (1970). This method involved fermentation of rapeseed presscake by *Geotrichum candidum*. During fermentation the fungus progressively rendered the rapeseed oil meal proteins soluble and hydrolyzed glucosinolates and isothiocyanates. Nutritional experiments on mice showed that the fermented RSOM possessed no toxicity and that the recovered protein meal gave greater gains in weight than soybean or peanut meals. More detailed toxicity studies are presently in progress.

Recent investigations by Eklund *et al*. (1971, 1971a) have produced a detoxified lipid-protein concentrate from rapeseed (*B. napus*) by a water-ethanol extraction method. The lipid-protein concentrate contained less than 0.4 mg/g of organic aglucons (glucosinolates) or not more than 5% of the content of organic aglucons present in raw rapeseed. This is lower than the corresponding value of Ballester *et al*. (1970) for a rapeseed meal preparation. The biological value and toxicological properties of the preparations were tested on growing male rats. PER

values were comparable to casein. No adverse pathological, histopathological, or haematological changes were observed. Further toxicological examinations, using both male and female rats as well as diets containing larger proportions of the lipid-protein concentrate, were proposed.

Statement of Objectives

From the literature survey it is apparent that the main use for rapeseed oil meal at the present time is in animal feeds. However, the utilization of RSOM as a protein supplement for animal feeds is limited by its digestibility, palatability and toxicity. The objective of this study is to investigate the use of rapeseed oil meal derivatives as substrates for the growth of microorganisms. In this way the problems mentioned above may be circumvented. Other oil seed meals (soybean, groundnut, cottonseed) are currently being used for this purpose. The initial approach will be the preparation of acid and enzymic hydrolyzates of RSOM and an evaluation of their ability to support the growth of *Candida utilis*. If rapeseed oil meal could be used in this way, it would be of considerable economic benefit to the rapeseed industry.

MATERIALS AND METHODS

Test Organism

a) Stock cultures and preparation of inoculum

Candida utilis ATCC 9950 (American Type Culture Collection, Rockville, Maryland, U.S.A.) was used throughout this study. Stock cultures were prepared from original freeze-dried cultures and maintained at 0°C by monthly sub-culture on Bacto-Sabouraud Dextrose agar slants (Difco Laboratories, Detroit, Michigan, U.S.A.). Bacto-Sabouraud Dextrose agar had the following composition (g/l):

Neopeptone, Difco	10.0
Bacto-dextrose	40.0
Bacto-agar	15.0
Final medium pH at 25°C	5.6

One hundred ml of Bacto-Sabouraud Dextrose Broth (Difco Laboratories) was dispensed into a 300 ml four-baffle culture flask (Bellco Glass Inc., Vineland, New Jersey, U.S.A.) and sterilized at 121°C for fifteen minutes. After autoclaving, the pH of the medium was 5.7. Bacto-Sabouraud Dextrose Broth had the following composition (g/l):

Neopeptone, Difco	10.0
Bacto-dextrose	20.0

A minimal quantity of sterile Bacto-Sabouraud Dextrose Broth (2 - 4 ml) was dispensed by a sterile disposable syringe (American Hospital Supply, Evanston, Illinois, U.S.A.) onto a stock culture slant and aseptically

transferred to Bacto-Sabouraud Dextrose liquid medium in baffled 300 ml culture flasks. The sub-cultures were maintained at 30^oC and shaken at 120 oscillations per minute in a New Brunswick Psycrotherm shaker incubator (New Brunswick Scientific Co., New Jersey, U.S.A.). At least five transfers were carried out before the sub-culture was removed for experimental use. All sub-culture transfers and transfers to experimental media were conducted when the absorbance of the inoculum had reached 1.0. Absorbance measurements were done in a Beckman DB-G grating spectrophotometer (Beckman Instruments, Inc., Fullerton, California, U.S.A.) at 660 nm using uninoculated medium as a reference solution. This procedure insured cells of a consistent physiological age (mid-logarithmic).

Samples of actively growing cultures were routinely removed and examined microscopically to assess possible bacterial contamination and yeast growth. Bacterial contamination was evaluated by the Gram-stain procedure (Harrigan and McCance, 1966). Lactophenol picric stain was used to examine yeast cells prior to "harvest" procedure.

Before transfer to 2 l experimental culture flasks, the complete contents of the Bacto-Sabouraud Dextrose Broth sub-culture flasks (cultures of O.D. 660 nm 1.0) were transferred to sterile, tared polypropylene centrifuge bottles and centrifuged at 8000 rev/min for 15 minutes in a RC2-B automatic superspeed refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conneticut, U.S.A.) at 0^oC and resuspended in phosphate buffer (0.2M $Na_2PO_4/Na_2H_2PO_4 \cdot H_2O$, pH 7.0), centrifuged and washed a second time, and finally resuspended in phosphate buffer to an absorbance of 1.0 at 660 nm.

b) Inoculum dry weight

Dry weight of inoculum was determined by weighing packed cells retained by a HA 0.45 μ Millipore filter (Millipore Corp., Montreal, Canada) after they were washed with distilled water and dried by 24 hours at 80°C under vacuum (Müller and Dawson, 1968) (Fig. 2).

c) Culture conditions

One litre of culture medium was dispensed into a two liter Erlenmeyer culture flask fitted with a special side arm for inoculation and sampling. The flask was allowed to equilibrate to 30°C prior to the addition of 1% (v/v) phosphate-washed cell suspension. The inoculum weight was usually 3 - 3.5 mg (dry weight). Cultures were incubated with agitation (120 oscillations per minute) at 30°C in a New Brunswick Psycrotherm shaker incubator.

Samples were periodically removed for measurement of growth.

d) Growth measurement

Growth was followed by conducting periodic measurements of absorbance of the yeast culture at 660 nm in a Beckman DB-G grating spectrophotometer using sterile, uninoculated medium as a reference solution. When necessary, dilutions of more dense samples were made in uninoculated medium.

Experimental Media

a) Commercial media

Two complex media (Sabouraud Dextrose Broth and Mycological Broth) were selected for the cultivation of *Candida utilis* ATCC 9950.

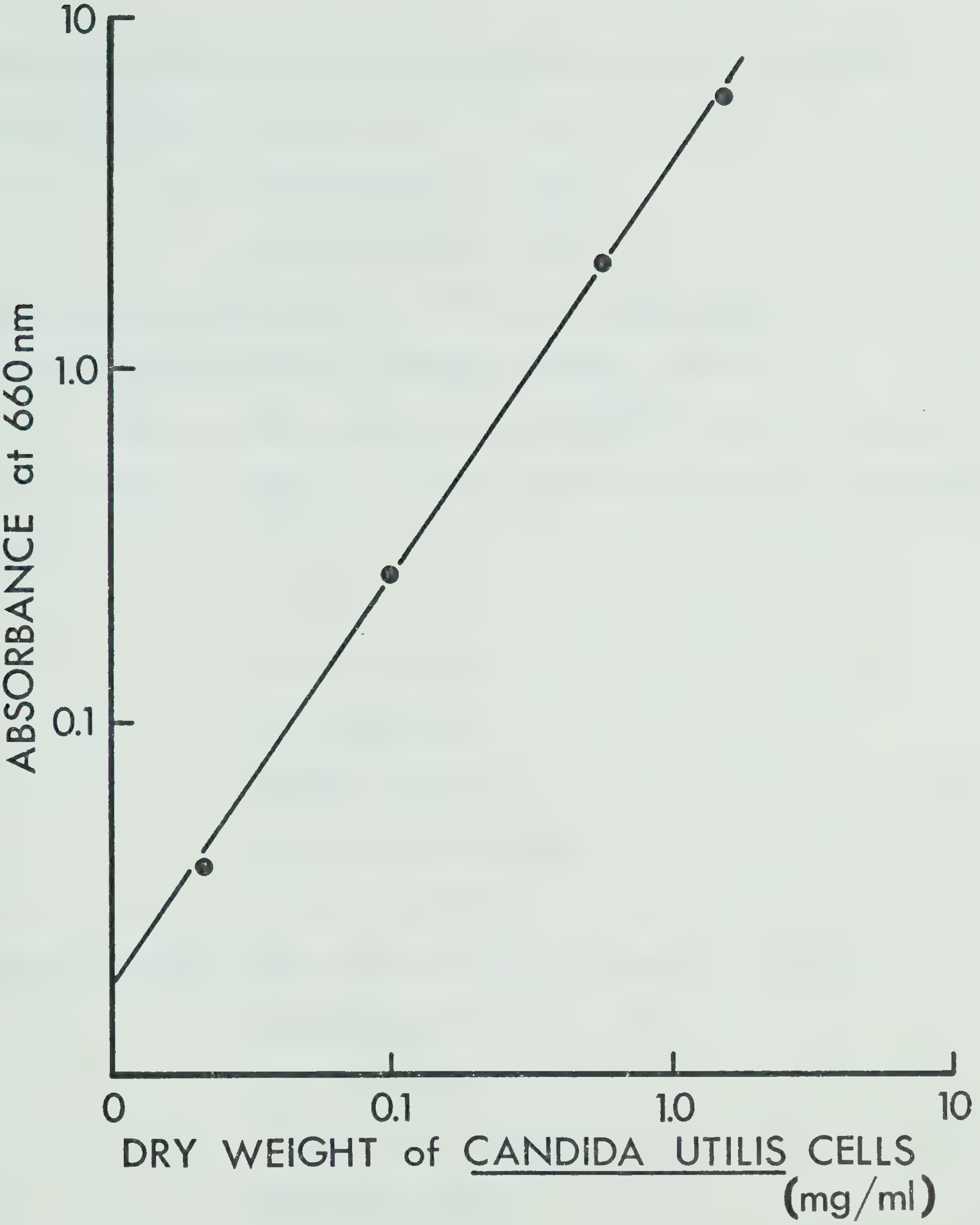

Fig. 2. Calibration Curve Relating Absorbance to Dry Weight of <u>Candida utilis</u>.

Bacto-Mycological Broth (Difco Laboratories) had the following composition (g/l):

Bacto-soytone	10
Bacto-dextrose	40

Both media were sterilized at 121°C for fifteen minutes and pH adjusted to 5.5 or 7.0 with sterile 1.0N and 0.1N HCl or NaOH.

Bacto-Tryptic Soy Broth and Czapek-Dox Broth (Difco Laboratories) were also used. Tryptic Soy Broth (TSB) had the following composition (g/l):

Bacto-tryptone	17
Bacto-soytone	3
Bacto-dextrose	2.5
Sodium chloride	5
Dipotassium phosphate	2.5
Final pH at 25°C	7.3

Czapek-Dox Broth (CDB) had the following composition (g/l):

Saccharose, Difco	30
Sodium nitrate	3
Dipotassium phosphate	1
Magnesium sulfate	0.5
Potassium chloride	0.5
Ferrous sulfate	0.01
Final pH at 25°C	7.3

Both media were sterilized in an autoclave for 15 minutes at 15 p.s.i. (121°C).

b) Basal medium

To determine nitrogen utilization, a chemically-defined synthetic medium was employed (Brown and Rose, 1969). The basal medium (without the nitrogen source) consisted of KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; glucose, 2.0 g; D-biotin, 0.195 pg; and distilled water to one liter. Glucose, salts, and nitrogen sources were sterilized individually. Glucose and salts (KH_2PO_4, $MgSO_4 \cdot 7H_2O$) were sterilized by autoclaving (121°C for fifteen minutes). The test nitrogen compounds (inorganic and organic) were added to the basal medium after sterilization (121°C for fifteen minutes) or Millipore filtration (0.45 μ). Urea was filter sterilized. D-biotin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was prepared by serial dilution using distilled water as diluent, filter sterilized, and aseptically added to Brown and Rose's medium. The pH of the medium was adjusted to 5.5 with sterile 1.0N and 5.0N HCl after sterilization and prior to inoculation.

The nitrogen compounds tested were: casein hydrolyzate (enzymatic, Nutritional Biochemicals Corp.); ammonium sulfate, ammonium nitrate, dibasic ammonium phosphate, sodium nitrate, sodium nitrite, potassium nitrate, potassium nitrite and urea (Fisher Scientific Co., Ltd., Fair Lawn, New Jersey, U.S.A.).

In addition, several commercial and laboratory digests (acid and enzyme hydrolyzates) were tested for their ability to provide sufficient carbon and nitrogen for the growth of Candida utilis. The following compounds were tested: Bacto-peptone, Bacto-soytone, Bacto-neopeptone, Bacto-proteose peptone, Bacto-tryptose, and Bacto-tryptone (Difco Laboratories). Laboratory digests included acid (HCl) and enzymic

(trypsin, pepsin) hydrolyzates of rapeseed oil meal. Total nitrogen content of the digests was determined by micro-Kjeldahl (A.O.A.C., 1955).

Each digest was added singularly to one liter distilled water to give an equivalent nitrogen concentration of 1000 mg nitrogen/l, sterilized at 121°C for fifteen minutes, allowed to cool, and pH adjusted to 5.5. In order to compensate for increased turbidity and sedimentation after sterilization at 1000 mg nitrogen/l, nitrogen concentration of rapeseed oil meal digests were reduced approximately ten-fold, 110 mg nitrogen/l. One per cent inoculum was added. Growth studies were carried out in shake culture (120 oscillations/minute) at 30°C.

c) Preparation of rapeseed oil meal hydrolyzates

Rapeseed oil meal was obtained from Western Canadian Seed Processors Ltd., Lethbridge, Alberta. Two hundred pounds of rapeseed oil meal was mixed in a Gosselin stainless steel churn (J.A. Gosselin Co. Ltd., Drummondville, Quebec, Canada) to achieve sample homogeneity, packaged in a polyethylene bag, placed in an air-tight fibreboard container and stored in a cold-room at 5°C. Samples to be used for experimental purposes were further homogenized in a Waring blender for five minutes.

Proximate analyses of rapeseed oil meal prior to preparation of hydrolyzates were conducted. Analyses included moisture, total nitrogen, oil, protein, ash, crude fibre, calcium, phosphorus, isothiocyanates, carbohydrate and chloride content.

i) Hydrochloric acid digestion (Reflux Method)

Rapeseed oil meal (150 g) was mixed with 1500 ml constant boiling 6.0N HCl (A.O.A.C., 1955) in a round bottom distillation flask (3 l). A Liebig condenser was attached to the distillation flask and used as a reflux column. To insure conditions of continuous digestion a variable transformer was used to regulate power input to the heating mantle. The mixture was refluxed for 24 hours. Aliquots of hydrolyzate (25 ml) were removed periodically, cooled in an ice bath, centrifuged at 7000 rev/min for 15 minutes (Sorvall RC2-B) and the supernatants filtered by vacuum through Whatman No. 4 filter paper to remove any particulate material.

Total nitrogen content of the supernatants was determined by micro-Kjeldahl method (A.O.A.C., 1955). Analyses were performed in triplicate.

Preparation of rapeseed oil meal hydrolyzate by the reflux method (RSOM-H) on a semi-pilot plant scale included the following procedures:

Rapeseed oil meal (300 g meal/2 l 6N HCl)
|
Reflux 24 hours
|
Cool in ice
|
Filter using vacuum (15 p.s.i.) through
Whatman No. 541 and No. 4 filter paper
|
Adjust filtrate pH to 6.0 using
1.0N and 10.0N NaOH
|
Freeze-dry 12 hours
|
Store in air-tight container at $0^{o}C$

Acid hydrolyzed rapeseed oil meal was lyophilized using a RePP 42-FFD-WS Sublimator (RePP Sublimator Division of Vir Tis Company Inc., Gardiner, New York, U.S.A.). Acid hydrolyzate was dispensed into stainless freeze-drying trays and prefrozen at -20^{0}F for three hours. The following conditions were maintained during lyophilization: condenser temperature -60^{0}F, shelf temperature 35^{0}C, vacuum 50 μ.

ii) Hydrochloric acid digestion (Autoclave Method)

Gladstone and Fildes (1940) studied the preparation of acid hydrolyzed casein. Casein was heated with strong hydrochloric acid until completely hydrolyzed to its constituent amino acids either by reflux with 6N HCl for 18 hours or autoclaved with 6N HCl for 45 minutes at 120^{0}C. This "autoclave" procedure merited consideration since it reduced hydrolysis time.

Preliminary investigations were conducted to evaluate optimum hydrolysis time prior to bulk preparation of hydrolyzate. Five grams of rapeseed oil meal was added to 99 ml Pyrex bacteriological dilution bottles containing 50 ml constant boiling 6N HCl. Hydrolysis was carried out at 121^{0}C for varying time intervals (5 - 120 minutes). Acid digests were filtered twice using vacuum through Whatman No. 42 filter paper. The following analyses were performed: total nitrogen, α-amino nitrogen, ammonia nitrogen and dry weight determination of acid-insoluble residue.

Bulk quantities of rapeseed oil meal hydrolyzate by the "autoclave" method were prepared using the following procedure:

Rapeseed oil meal (50 g meal/500 ml 6N HCl)

Autoclave 30 minutes at 121°C

Cool in ice bath

Vacuum filtration through Whatman No. 42 (twice)

Adjust pH to 5.0 using 1.0N and 10.0N NaOH

Freeze-dry for 5 hours

Store in air-tight container at 0°C

Conditions of freeze-drying were similar to those used in preparation of rapeseed oil meal hydrolyzate by the "reflux" method.

iii) Pepsin digestion

Enzymic hydrolysis of rapeseed oil meal using pepsin was investigated. A preliminary experiment was conducted to evaluate the feasibility of pepsin digestion prior to bulk preparation. Two grams of rapeseed oil meal was added to a 250 ml Erlenmeyer flask containing 100 ml acid solution (hydrochloric acid in deionized water, pH 1.50). The mixture was allowed to equilibrate at 40°C for 30 minutes prior to the addition of enzyme. A stock solution of pepsin (N.F. grade, Fisher Scientific) was prepared by dissolving one gram pepsin in 100 ml acid solution (pH 1.50). Pepsin was added at three concentrations: 0.5%, 2.5%, 3.75% (w/w, enzyme/substrate). A control flask containing no enzyme was also prepared. Proteolysis was carried out in a New Brunswick Metabolyte Water Bath Shaker (New Brunswick Scientific Co., Inc.) at 40°C

with agitation (300 rev/min). Aliquots (5 ml) were periodically removed, centrifuged at 5000 rev/min for 10 minutes and total nitrogen content of the supernatant determined by micro-Kjeldahl methods. Analyses were conducted in triplicate. Results were expressed as percentage nitrogen solubilized, that is, nitrogen content of supernatant.

A second experiment was conducted to monitor pepsin digestion as a function of time. Rapeseed oil meal (100 g) was added to an Erlenmeyer flask (2 l) containing one l acid solution (pH 1.50). The Erlenmeyer flask was immersed in a temperature controlled 12" x 12" x 12" perspex (Plexiglas) tank. A Haake thermoregulator (Model E 72, Fisher Scientific) immersed in the tank maintained a constant temperature (40 ±0.02^{0}C). Agitation at 600 rev/min was provided by a magnetic stirring bar activated by placing the perspex tank plus Erlenmeyer flask on a non-heating magnetic stirrer (Bellco Glass Inc.). A stroboscope (Strobotac 1531-A, General Radio Co., Concord, Massachusetts, U.S.A.) was used to determine the rate of rotation of the stirring bar. The flask was allowed to equilibrate for 30 minutes to the desired temperature and pepsin added (1%, w/w). Two milliliters of toluene was added to prevent bacterial growth. Samples were removed at precise intervals, centrifuged at 9000 rev/min for 15 minutes and stored at 0^{0}C until required. Total nitrogen and ammonia nitrogen analyses were performed on the supernatants.

To achieve substantial quantities of pepsin digested rapeseed oil meal, the following procedure was adopted:

Rapeseed oil meal (500 g meal/5 l acid solution, pH 1.50)

Temperature equilibration (40°C, 30 minutes)

Add 10 ml toluene

Pepsin digestion for 24 hours
(One per cent pepsin added, w/w)

Filtration through cheesecloth using
hydraulic press (2X)

Filtrate centrifugation (9000 rev/min/15 minutes)

Adjust pH to 5.0

Freeze-dry for 24 hours

Store in air-tight container at 0°C

Bulk preparation of pepsin digested rapeseed oil meal (RSOM-P) was conducted in a 7.5 l Microferm Laboratory Fermentor (New Brunswick Scientific Co., Inc.). Efficient agitation was achieved by a pair of impellers with flat stirring paddles and by four vertical baffles within the fermentation vessel. Impeller speed was maintained at 600 rev/min throughout the study. Toluene (10 ml) was added to protect the digest from bacterial spoilage.

Conditions of freeze-drying were similar to those used in the preparation of acid hydrolyzates of rapeseed oil meal.

iv) Trypsin digestion

A tryptic digest of rapeseed oil meal was also prepared.

Experimental conditions were similar to those used in pepsin digestion. Recognizing the significance of pH on enzyme activity, rapeseed oil meal (100 g) was added to a 2 l Erlenmeyer flask containing one liter 0.1M phosphate buffer ($NaH_2PO_4 \cdot 7H_2O/Na_2HPO_4$, pH 8.0) or 1 l deionized distilled water (pH 8.0) and allowed to equilibrate at 25°C for 30 minutes prior to addition of one gram trypsin powder (purified 1:80, Fisher Scientific). Digestion temperature was controlled at 25°C with agitation at 600 revolutions per minute. Two milliliters of toluene was added to prevent microbial contamination. Samples were withdrawn periodically and centrifuged at 9000 rev/min for 15 minutes. Supernatants were analyzed in triplicate for total nitrogen and ammonia nitrogen.

To accommodate bulk preparation, 7.5 l Microferm Laboratory Fermentors were used. Operational conditions were similar to those used in preparation of pepsin digested rapeseed oil meal. Large quantities of trypsin digest rapeseed oil meal (RSOM-T) were prepared by the following procedure:

Rapeseed oil meal
(500 g meal/5 l phosphate buffer, pH 8.0)

Temperature equilibration (25°C, 30 minutes)

Add 10 ml toluene

Trypsin digestion for 24 hours
(One per cent trypsin added, w/w)

Filtration through cheesecloth using
hydraulic press (2X)

Filtrate centrifugation
(9000 rev/min for 15 minutes)

No pH adjustment (pH 7.0)

Freeze-dry 24 hours

Store in air-tight container at 0°C

Methods of Analysis

a) Oil

Oil content of rapeseed oil meal and freeze-dried hydrolyzates (2 g) was estimated by Soxhlet extraction with n-hexane (200 ml) for 8 hours. Longer periods of extraction failed to extract significant amounts of residual oil.

b) Moisture

One to two grams of sample was weighed into a covered, tared aluminum dish and placed in a Fisher Isotemp oven (Fisher Scientific Co. Ltd., Montreal, Quebec, Canada) at 110°C for 6 hours. At the end of this time, the dish was removed, transferred to a desiccator at room temperature, and allowed to cool prior to weighing to constant weight. Moisture content was reported as loss in weight.

c) Crude fiber

Crude fiber was determined as loss in weight on ignition ($600^{\circ} \pm 15^{\circ}$C for 30 minutes) of dried residue remaining after digestion of oil-free sample with 1.25% H_2SO_4 and 1.25% NaOH solutions under specific conditions (A.O.A.C., 1965).

d) Ash

Total ash content was determined by modified A.O.A.C. method (1965). Rapeseed oil meal (2 g) was incinerated at 600°C for 2 hours in a muffle furnace (Linberg, Hevi-Duty Heating Equipment Company, Waterton, Wisconsin, U.S.A.). Constant temperature was maintained in the muffle furnace by an automatic control pyrometer. These conditions resulted in ash uniform in color, white or gray, and free from particles of unburned carbon and fused material.

e) Calcium and Phosphorus

Calcium and phosphorus analysis of rapeseed oil meal was conducted spectrophotometrically using a Technicon Auto Analyzer (Technicon Instruments Corp., Inc., Ardsley (Chauncey), New York, New York, U.S.A.).

Rapeseed oil meal (1.0 ± 0.1 g) was digested with 12 ml nitric-perchloric acid mixture (2:1 v/v), cooled and diluted to appropriate volume with deionized distilled water prior to calcium and phosphorus analysis.

The estimation of calcium was based on the stable color complex formed between calcium and o-cresol phthalein complexone or metaphtalein (2,6-xylenolphtalein-α,α'-bis (imino) diacetic acid) under alkaline conditions, pH 10.7 (Pollard and Martin, 1956). The developed color was measured at 580 nm in a flow cell 8 mm light path. A standard curve was prepared using calcium carbonate.

Phosphorus was determined by the vanado-molybdate method as described by Mission (1908), Koenig and Johnson (1942), Kitson and Mellon (1944). This method is based on the formation of a yellow color

(molybdivanadophosphoric acid) on addition of excess molybdate solution to an acidified solution mixture of a vanadate and an orthophosphate. The developed color was measured at a wavelength of 420 nm in a flow cell of 15 mm light path. A standard curve was prepared using potassium dihydrogen phosphate.

Calcium and phosphorus analysis of rapeseed oil meal was conducted by the Alberta Department of Agriculture, Soil and Feed Testing Laboratory, Edmonton, Alberta.

f) Chloride

Chloride was determined by the indirect Volhard method. The indirect method involves the addition of a known, excess amount of silver nitrate to the chloride-containing solution, removal of the precipitate formed, and titration of the remaining silver ion with standard thiocyanate solution.

Nitric acid (1 ml) and ferric ammonium sulphate (5 ml) were added to moisture-free sample (0.3 - 0.5 g) dissolved in 100 ml deionized distilled water. Excess 0.1 silver nitrate (35 ml) and 15 ml nitrobenzene were added. The mixture was vigorously shaken and excess silver nitrate remaining in solution was titrated with standardized 0.1N ammonium thiocyanate. Chloride content was calculated as percentage.

g) Isothiocyanate

Isothiocyanate analysis was performed according to a modified argentimetric method of Wetter (1955). This method involves the reaction of "mustard oil" (isothiocyanates) with ammonia to form a substituted thiourea which decomposes in ammoniacal silver nitrate forming insoluble

silver sulphide and a monosubstituted carbodiimide. The unreacted silver is determined volumetrically by the Volhard method.

Myrosinase digestion of rapeseed oil meal and rapeseed oil meal preparations was omitted. Distillation apparatus as described by Wetter (1955) was used throughout the analysis. Sample (2 g) was added to a 500 ml Erlenmeyer flask containing 100 ml 0.2 M citrate buffer (pH 4.0) and 10 ml 95% ethanol and steam distilled until approximately 70 ml of distillate was collected in a 125 ml receiving flask. The 125 ml receiving flask, containing 10 ml 0.1N silver nitrate and 2.5 ml 10% ammonium hydroxide, was immersed in an ice-water mixture to ensure rapid cooling. After distillation the receiving flask was fitted to an air reflux condenser, heated in a boiling water bath for 30 minutes, cooled, made up to a volume of 100 ml, and filtered through Whatman 42 filter paper. To 25 ml aliquots of the filtrate, 1 ml 6N nitric acid and 0.5 ml ferric ammonium sulphate was added. This mixture was titrated with 0.01N potassium thiocyanate and the isothiocyanate calculated as allylcarbinyl isothiocyanate.

Kjaer et al. (1953) and Ettlinger and Hodgkins (1954) reported the main component of "mustard oil" in rapeseed to be allylcarbinyl isothiocyanate.

h) Carbohydrate

Total carbohydrate content was estimated by the colorimetric method of Fales (1951). A suitable dilution of oil-free sample was mixed with 5 ml anthrone solution, heated for 10 minutes in a boiling water bath and the intensity of the blue color read at 620 nm on a

Beckman DB-G grating spectrophotometer. The concentration of carbohydrate was calculated from a standard curve prepared with D-glucose (Fisher Scientific Co., Ltd.) (Fig. 3).

Sugars were extracted from defatted rapeseed oil meal by thorough maceration in a Waring blendor in 100 ml of boiling 80% (v/v) ethanol. After 30 minutes of heating the alcohol was decanted and the residue extracted twice with 80% ethanol. The combined extracts were diluted to appropriate volume (500 ml) with 80% ethanol. Total carbohydrate content of the ethanol extract was determined by the anthrone-sulphuric acid method.

i) Total nitrogen

Total nitrogen content of rapeseed oil meal and rapeseed oil meal hydrolyzates was determined by the micro-Kjeldahl method (A.O.A.C., 1955) with potassium sulphate-mercuric oxide as a catalyst and methyl red-bromcresol green as indicator. All solutions were made with distilled deionized water and analytical grade reagents. A modified Parnas-Wagner distillation apparatus was used for steam generation and distillation. The apparatus used is shown in Fig. 4.

Non liquid samples (meal, hydrolyzates) were extracted with n-hexane (b.p. range 67 - 68°C) in a Soxhlet apparatus for 12 hours and allowed to dry in air overnight prior to analysis.

Crude protein content of rapeseed oil meal (N x 6.25) was determined by the micro-Kjeldahl method.

j) Ammonia nitrogen

Ammonia nitrogen was determined by modified micro-Kjeldahl

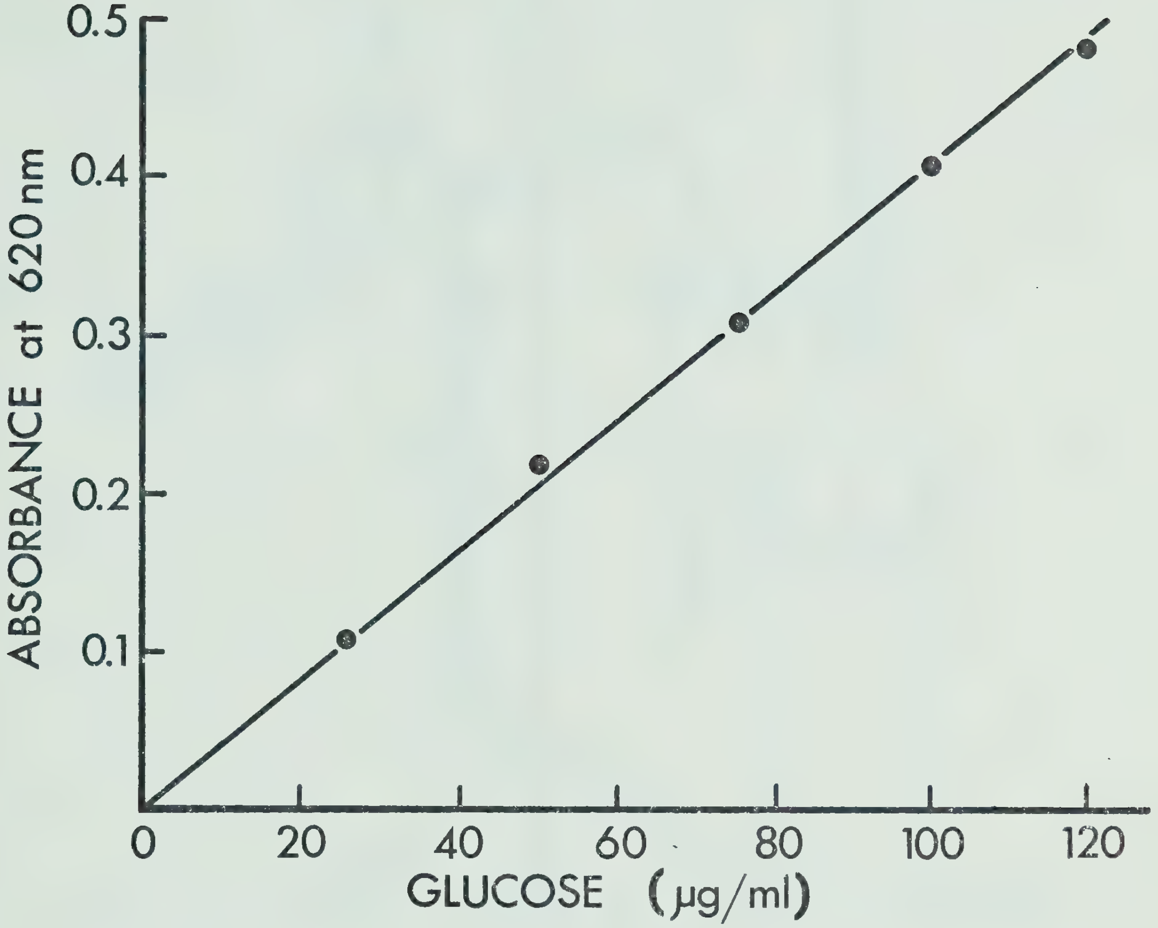

Fig. 3. Standard Curve for Determination of Total Carbohydrate.

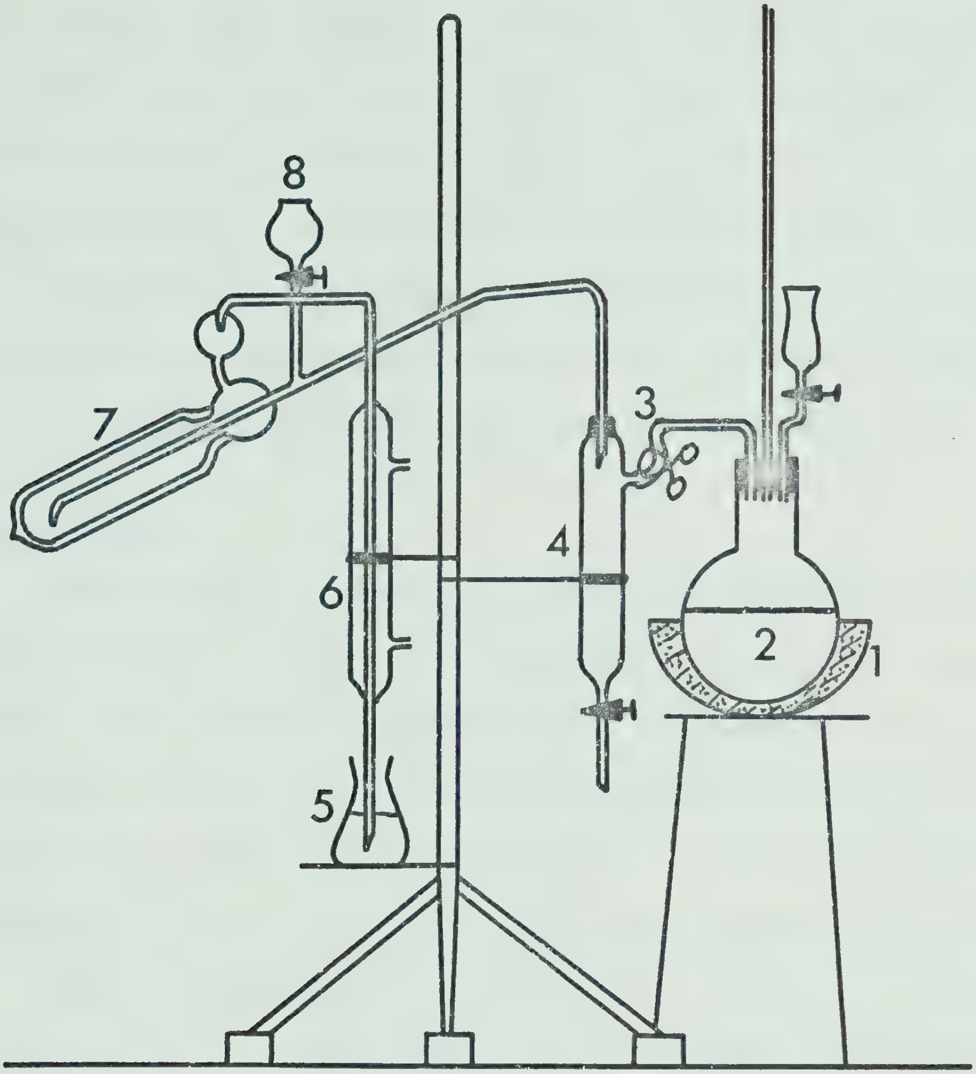

Fig. 4. Modified Parnas-Wagner Distillation Apparatus

1. Heating mantle
2. Steam generator
3. Pinch clamp for self-cleaning operation
4. Steam trap
5. Receiving flask
6. Condenser
7. Distillation tube
8. Sample funnel connected to distillation tube

method. Sulphuric acid digestion was omitted. Suitable dilutions of hydrolyzate or digest were steam distilled (Parnas-Wagner distillation apparatus) with 10 ml 50% NaOH-5% $Na_2S_2O_3 \cdot 5\ H_2O$ into 5 ml 4% boric acid solution containing 2 - 3 drops methyl red-bromcresol green indicator. The boric acid solution (approximately 15 ml distillate collected and diluted to 50 ml with deionized distilled water) was titrated with standardized 0.02N HCl and the nitrogen content calculated.

k) Amino nitrogen

Amino nitrogen content was determined by the method of Yemm and Cocking (1955). This colorimetric method is based on the formation of a purple color (diketohydrindylidenediketohydrindamine, DYDA) by the stoicheiometric reaction of triketohydrindene hydrate (ninhydrin) and most amino acids. Yemm and Cocking found that all the amino acids studied gave quantitative yields of DYDA except tryptophan and lysine.

One ml of amino acid solution (0.05 - 5.6 μg amino nitrogen) was mixed with 0.5 ml citrate buffer, pH 5 (0.2 M) and 1.2 ml potassium cyanide-methyl Cellosolve-ninhydrin solution. The mixture was heated for 15 minutes in a boiling water bath, cooled for 5 minutes in running tap water, made up to convenient volume with 60% ethanol and the intensity of the purple color read at 570 nm on a Beckman DB-G grating spectrophotometer. Amino nitrogen concentrations were calculated from a standard curve using L-leucine (Calbiochem, La Jolla, California, U.S.A.) (Fig. 5).

This procedure has several advantages over that of Moore and Stein (1948): greater sensitivity, lower sensitivity to ammonia, shorter

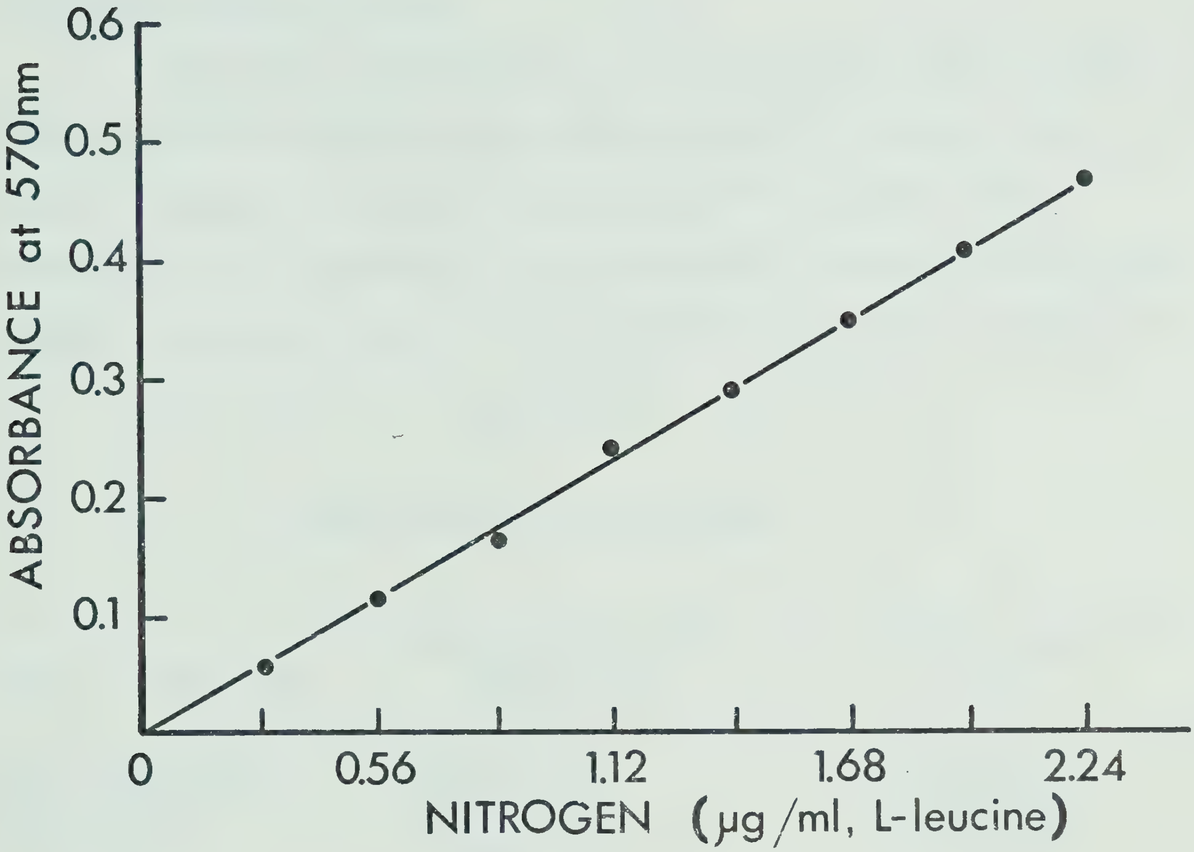

Fig. 5. Standard Curve for the Determination of α-Amino Nitrogen.

and less critical heating time, ninhydrin economy and avoids the complications of having to store reduced ninhydrin under nitrogen.

1) Soluble free amino acids

Quantitative determination of the amino acid content of RSOM preparations was conducted with a Beckman Model 121 automatic amino acid analyzer (Beckman Instruments, Inc., Spinco Division, Palo Alto, California, U.S.A.) operated in conjunction with a Beckman 125 Integrator (Beckman Instruments, Inc.). Analysis conditions are shown in Table 11.

TABLE 11

AMINO ACID ANALYSIS CONDITIONS

Analysis Conditions	Acidic and Neutral Amino Acids	Basic Amino Acids
Resin nomenclature	Beckman UR30	Beckman PA35
Column dimensions	0.9 x 69 cm	0.9 x 23 cm
Resin height	0.9 x 56 cm	0.9 x 10 cm
Column temp.	52^{o}C	52^{o}C
Buffer (sodium citrate)	1st pH 3.00 (50 min.) 2nd pH 3.44 (40 min.) 3rd pH 4.35 (90 min.)	4th pH 5.25 (100 min.)
Flow rate		
Buffer	70 ml/hr	70 ml/hr
Ninhydrin	35 ml/hr	35 ml/hr
Sodium Citrate Buffer		
1st Buffer	0.2N Na-citrate pH 3.00	
2nd Buffer	0.2N Na-citrate pH 3.44	
3rd Buffer	0.2N Na-citrate pH 4.35	
4th Buffer	0.35N Na-citrate pH 5.25	

Rapeseed oil meal hydrolyzates (2 - 3 mg) were dissolved in 5 - 10 ml 0.2N sodium citrate buffer (pH 2.2) and 568 λ was used for the determination of acidic, neutral and basic amino acids. Amino acid composition of RSOM preparations was reported as g amino acid/g nitrogen.

m) Dry weight of acid-insoluble residue

Dry weight of acid-insoluble residue remaining after 6N HCl hydrolysis at 121°C was determined by weighing the residue retained by Whatman No. 42 filter paper after washing 2 - 3 times with distilled water and dried for 24 hours at 80°C under vacuum.

RESULTS

Rapeseed Oil Meal Digests

Preliminary experiments on digestion of RSOM with 6N HCl showed that approximately 90% of the nitrogenous components in RSOM were hydrolyzed within one hour (Table 12). Bulk preparations of rapeseed oil meal by the reflux method (RSOM-H) involved acid digestion for 24 hours. After 24 hours, 97% of the total nitrogen content of rapeseed oil meal was hydrolyzed. This procedure was employed to be representative of conventional hydrolyzate preparation, i.e., rapeseed oil meal protein would be degraded into constitutive amino acids. The total nitrogen content of RSOM-H was 1.60%.

In order to evaluate the feasibility of the production of a rapeseed oil meal hydrolyzate using 6N HCl by the "autoclave" method, it was necessary to establish experimental conditions whereby maximum rapeseed protein was digested. Table 13 and Fig. 6 show acid digestion of rapeseed oil meal at 121°C for different periods of time. Efficiency of hydrolysis was determined by total nitrogen and α-amino nitrogen content of the filtrate. Maximum hydrolysis was attained after 60 minutes at 121°C; however, shorter periods of hydrolysis produced filtrates similar in total nitrogen and α-amino nitrogen content. After 30 or 45 minutes at 121°C, efficiency of hydrolysis was approximately 80% while an additional 15 minutes only increased hydrolysis efficiency 2%.

TABLE 12

HYDROLYSIS OF RAPESEED OIL MEAL WITH 6N HCl BY THE REFLUX METHOD

Period of Hydrolyses (Hours)	Nitrogen Content of Filtrate (g nitrogen/g RSOM)	Efficiency of Hydrolysis $\left(\frac{\text{Nitrogen in filtrate}}{\text{Original nitrogen in RSOM}} \times 100\right)$
1	7.10	86.8
2	7.45	91.1
3	7.45	91.1
6	7.68	93.9
9	7.90	96.6
12	7.94	97.1
24	7.74	94.6
36	7.84	95.9

TABLE 13

HYDROLYSIS OF RAPESEED OIL MEAL WITH 6N HCl BY THE AUTOCLAVE METHOD

Period of Hydrolysis (Min at 121°C)	Weight of Acid-Insoluble Residue (g)	Total Nitrogen Content of Acid-Insoluble Residue (mg N/g residue)
5	1.47	29.6
15	1.49	20.5
30	1.46	23.7
45	1.44	21.9
60	1.42	19.5
120	1.42	18.2

TABLE 13 -- Continued

Free Ammonia Nitrogen Content of Hydrolyzate (mg N/5 g RSOM)	α-Amino Nitrogen Content of Hydrolyzate (mg N/5 g RSOM)	Total Nitrogen Content of Hydrolyzate (mg N/5 g RSOM)	Efficiency of Hydrolysis Total N (Hydrolyzate) / Total N (RSOM)
28.1	83.7	219.0	80.2
27.5	99.0	209.3	76.7
28.2	109.3	217.6	79.7
28.1	119.3	217.5	79.7
28.5	122.9	223.7	81.9
28.8	135.1	232.7	85.2

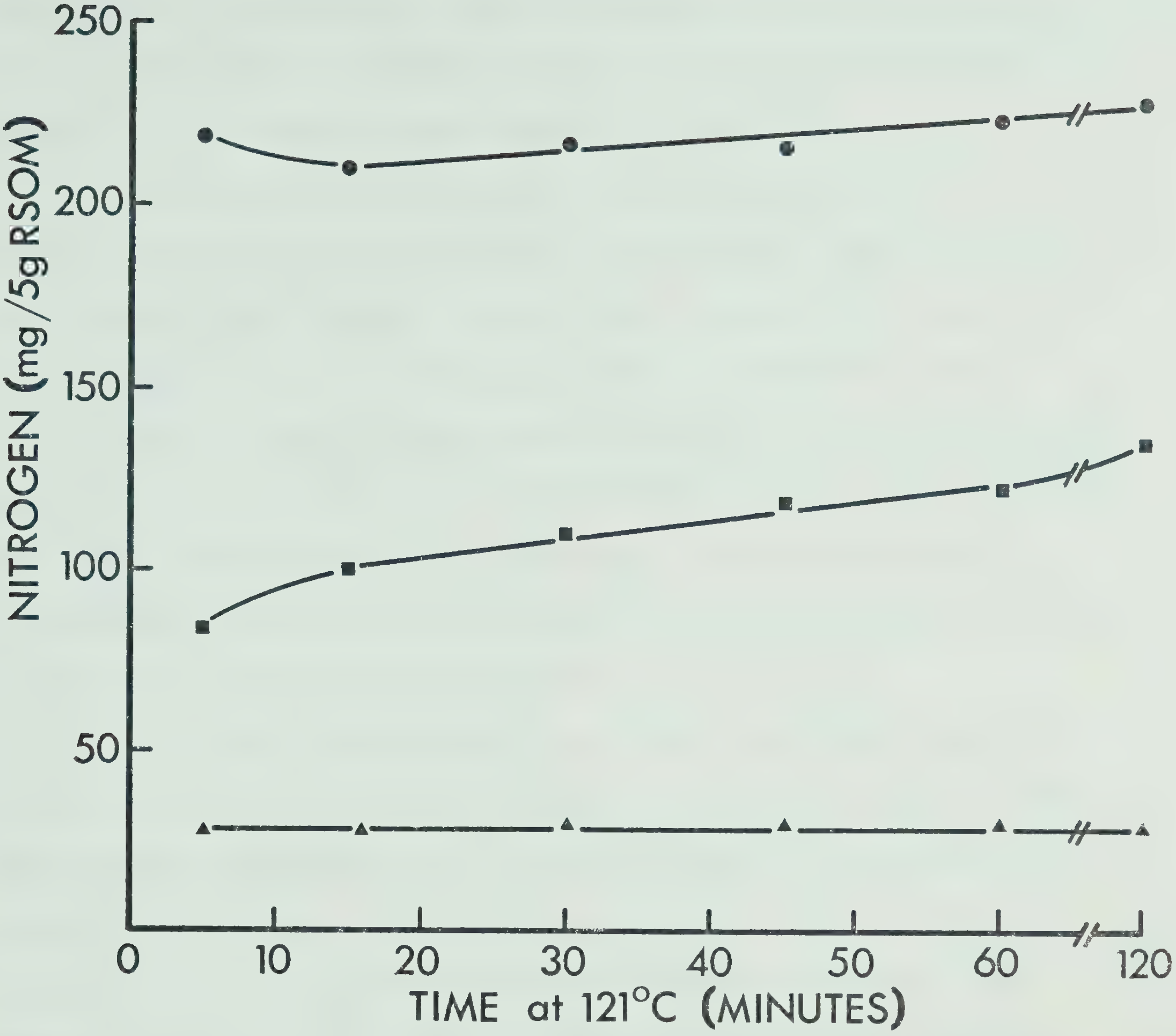

Fig. 6. Hydrolysis of Rapeseed Oil Meal with 6N HCl by the Autoclave Method (Nitrogen content).

Total nitrogen ●—● , α-Amino nitrogen ■—■ ,

Free ammonia nitrogen ▲—▲ .

Consequently it was decided to select conditions of 30 minutes at 121°C for bulk preparation of rapeseed oil meal by the "autoclave" method (RSOM-HA^{30}). The total nitrogen content of RSOM-HA^{30} was 1.10%.

The preliminary experiment to determine optimum pepsin concentration for preparation of RSOM digests showed a direct relationship between initial reaction rate and pepsin concentration. However, maximum solubilization of nitrogenous material (approximately 75%) occurred after 24 hours at enzyme concentration 0.5 - 2.5% (w/w). It was considered that the reduced level of solubilized nitrogen (60%) associated with 3.75% pepsin was due to auto-digestion. In view of the above, a 1% (w/w) concentration of pepsin was used in subsequent studies. Trypsin was also used at 1% (w/w) concentration.

The results of the digestion of RSOM by pepsin and trypsin in buffered and non-buffered medium is shown in Table 14 and Fig. 7. Since the pH optimum for pepsin is 2.0 and after 48 hours of digestion the pH of the filtrate had only risen to 3.30, no buffer system was selected to be used in bulk preparations of pepsin digested rapeseed oil meal. However, this was not the case with trypsin (optimum pH 8.0). Because of changes in pH during trypsin digestion in non-buffered medium, only 48% of the total nitrogenous material had been solubilized after 48 hours. During the same period of time the pH of the digestion mixture had fallen from 8.00 to 4.80, thereby creating non-optimum conditions of enzyme digestion. In contrast, when a phosphate buffer medium was used to stabilize fluctuations in pH, the quantity of nitrogen solubilized was 71% after 48 hours. After 24 hours, the quantity of nitrogen solubilized in the buffer system was 66%; consequently, bulk preparations

TABLE 14

CHANGES IN pH AND NITROGEN DISTRIBUTION OF ENZYMIC DIGESTS OF RAPESEED OIL MEAL

Enzyme	Digestion Time (H)	Ammonia-Nitrogen in Digest (mg N/100 g RSOM)	Total Nitrogen in Digest (mg N/100 g RSOM)	Nitrogen Solubilized (%) $\frac{\text{Nitrogen Content of Digest}}{\text{Nitrogen Content of RSOM}} \times 100$	pH of Digestion Mixture
PEPSIN (1% w/w)	0	372	1350	24.8	-
	1	445	2560	47.0	3.20
	3	446	2630	48.3	3.20
	5	448	2830	51.9	3.20
	7	456	2840	52.1	3.30
	12	470	2960	54.3	3.20
	18	490	3100	56.9	3.30
	24	484	3200	58.7	3.35
	36	498	3220	59.1	3.25
	48	501	3290	60.1	3.30
TRYPSIN-Buffered (1% w/w)	0	322	2060	37.8	-
	1	328	2420	44.0	7.50
	3	-	3150	57.8	7.00
	6	414	3153	57.9	7.00
	9	423	3260	59.8	7.00
	12	428	3265	59.9	7.00
	18	445	3400	62.4	7.00
	24	476	3570	65.5	7.00
	35	490	3710	68.1	7.10
	48	512	3850	70.6	7.10
TRYPSIN-Non-buffered (1% w/w)	0	-	-	-	-
	1	260	2170	39.8	5.70
	3	294	2170	39.8	5.80
	6	311	2310	42.4	6.00
	9	319	2420	44.4	5.85
	12	333	2650	48.6	5.90
	24	442	2580	47.3	5.10
	36	454	2520	46.2	4.90
	48	476	2590	47.5	4.80

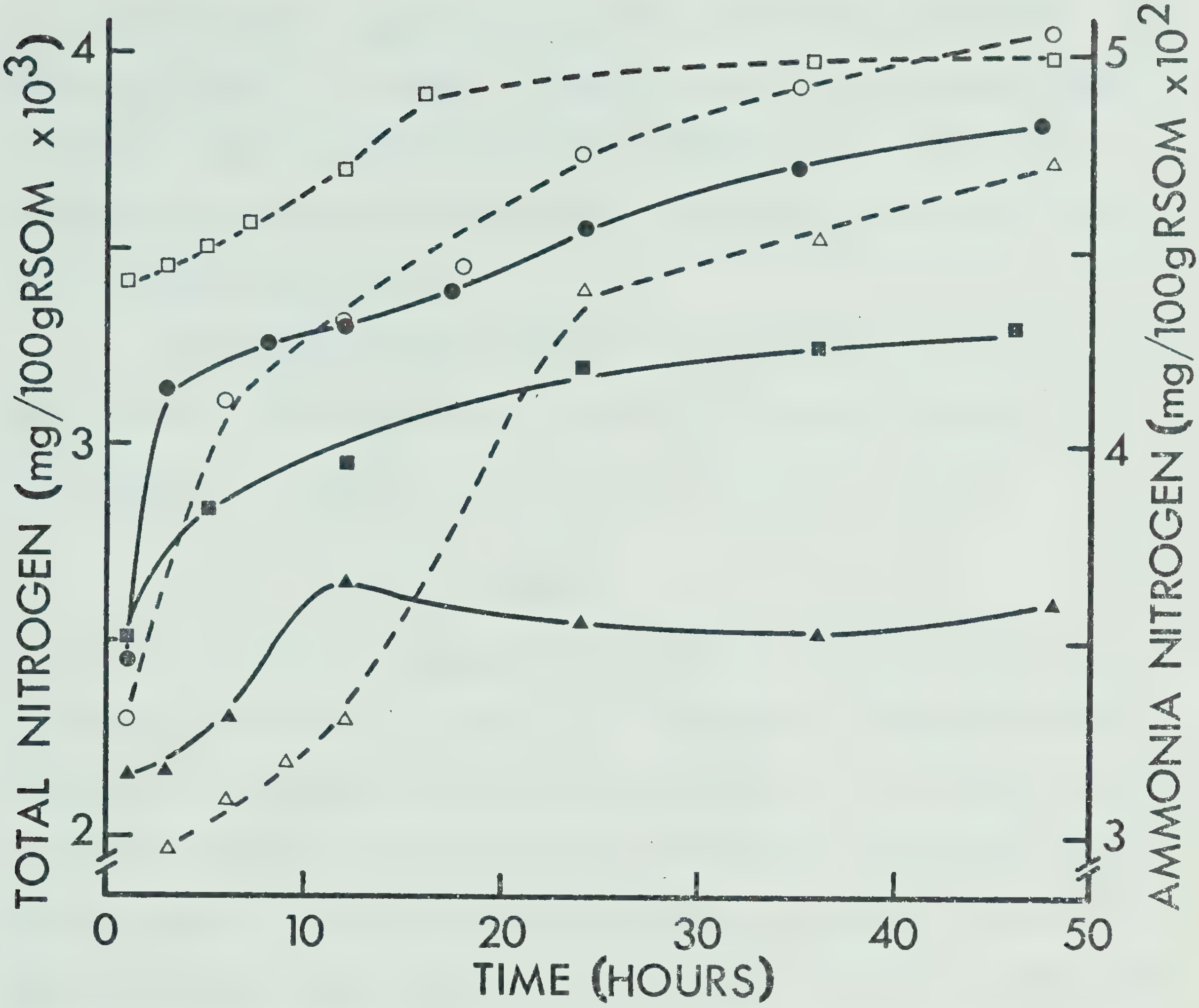

Fig. 7. Nitrogen Distribution of Enzymic Digests of Rapeseed Oil Meal.

Pepsin (ammonia nitrogen) □— —□ ,

Pepsin (total nitrogen) ■—■ ,

Trypsin (phosphate buffer, ammonia nitrogen) ○— —○,

Trypsin (phosphate buffer, total nitrogen) ●—● ,

Trypsin (no phosphate buffer, ammonia nitrogen) △— —△ ,

Trypsin (no phosphate buffer, total nitrogen) ▲—▲ .

of trypsin digested rapeseed oil meal were conducted in phosphate buffer (pH 8.00) for 24 hours. Longer periods of digestion were not used in an attempt to minimize trypsin auto-digestion. The total nitrogen content of tryptic and peptic rapeseed oil meal digests was 8.30 and 6.88% respectively.

Proximate analysis of RSOM and various acid and enzymic hydrolyzates of RSOM are shown in Table 15. Amino acid analysis of RSOM digest and commercial protein digest is shown in Table 16.

Growth

The growth of Candida utilis 9950 on a variety of nitrogen sources is illustrated in Figs. 8 - 12. Initially, experiments were conducted to evaluate the nitrogen requirements of this organism prior to the formulation of rapeseed oil meal hydrolyzates or digests into suitable media. Three conventional nitrogen sources were selected: ammonium sulphate, urea, and casein hydrolyzate. Ammonium sulphate and urea were chosen as simple sources of nitrogen, moreover, these compounds are generally used in industrial fermentations as they are inexpensive and readily available. Incorporation of urea as a nitrogen source in industrial operation is limited by its heat lability, i.e., it must be filter sterilized. This additional unit operation may be expensive and time consuming.

The relationship between ammonium sulphate concentration and growth response by Candida utilis is shown in Fig. 8. Increasing nitrogen concentration produced only slight variations in growth. After 30 hours, yeast yields were similar and independent of ammonium sulphate

TABLE 15

PROXIMATE ANALYSIS (Percentage) OF RAPESEED OIL MEAL AND RAPESEED OIL MEAL HYDROLYZATES

	Rapeseed Oil Meal (RSOM)	RSOM-H[1]	RSOM-HA30[2]	RSOM-P[3]	RSOM-T[4]
Moisture	7.39	4.56	3.73	8.07	7.21
Total Nitrogen	5.45	1.60	1.10	6.88	8.20
Fat	1.59	0.30	0.52	0.11	0.68
Chloride	3.55	40.22	38.48	4.13	3.16
Isothiocyanates	0.198	0.136	0.119	0.164	0.430
Carbohydrate	3.0	0.0	0.5	20.0	26.0
α-Amino Nitrogen		1.12	0.58	0.55	1.29
Peptone Nitrogen*		70.0	52.7	7.99	1.55
Protein (Nx6.25)	34.06				
Ash	7.29				
Crude Fibre	12.4				
Calcium (dry wt basis)	0.75				
Phosphorus (dry wt basis)	1.17				

[1] RSOM-H - Reflux method digest

[2] RSOM-HA30 - Autoclave method digest

[3] RSOM-P - Peptic digest

[4] RSOM-T - Tryptic digest

*Peptone Nitrogen = $\frac{\alpha\text{-Amino Nitrogen}}{\text{Total Nitrogen}} \times 100$

TABLE 16

AMINO ACID ANALYSIS OF PROTEIN DIGESTS

Amino Acid (g Amino Acid/ g Nitrogen)	RSOM-H	RSOM-HA[30]	RSOM-T	RSOM-P	Bacto-Soytone*	Bacto-Peptone*	Bacto-Neopeptone*
Lysine	.3950	.1945	.1743	.0038	.4000	.2903	.4482
Histidine	.1198	.0585	.0301	.0004	.1052	.0645	.1379
Ammonia							
Arginine	.3811	.2008	.1993		.4210	.5333	.3448
Aspartic Acid	.5089	.3504	.0129	.0165	.5263	.4000	.4827
Threonine	.3109	.1291	.0140	.0008	.2105	.1000	.2758
Serine	.3091	.2179	.0692	.0089			
Glutamic Acid	1.3469	.7747	.0541	.0383	1.1578	.7333	1.0344
Proline	.5397	.2598		.0058			
Glycine	.3202	.2392	.0031	.0014	.2105	1.5333	.4482
Alanine	.3193	.2168	.0241	.0082			
Half Cystine	.0663						
Valine	.3359	.1037	.0284	.0053	.1894	.2000	.4137
Methionine	.1152	.0594	.0300	.0047	.0526	.0666	.1724
Isoleucine	.2676	.0696	.0327	.0051	.2421	.1333	.3103
Leucine	.4991	.2379	.1092	.0157	.4210	.2333	.5862
Tyrosine	.1299	.0641	.1030	.0059	.2105	.0666	.3793
Phenylalanine	.2733	.1169	.0687	.0186	.3684	.1666	.3103

*Values calculated from data supplied by Difco Laboratories.

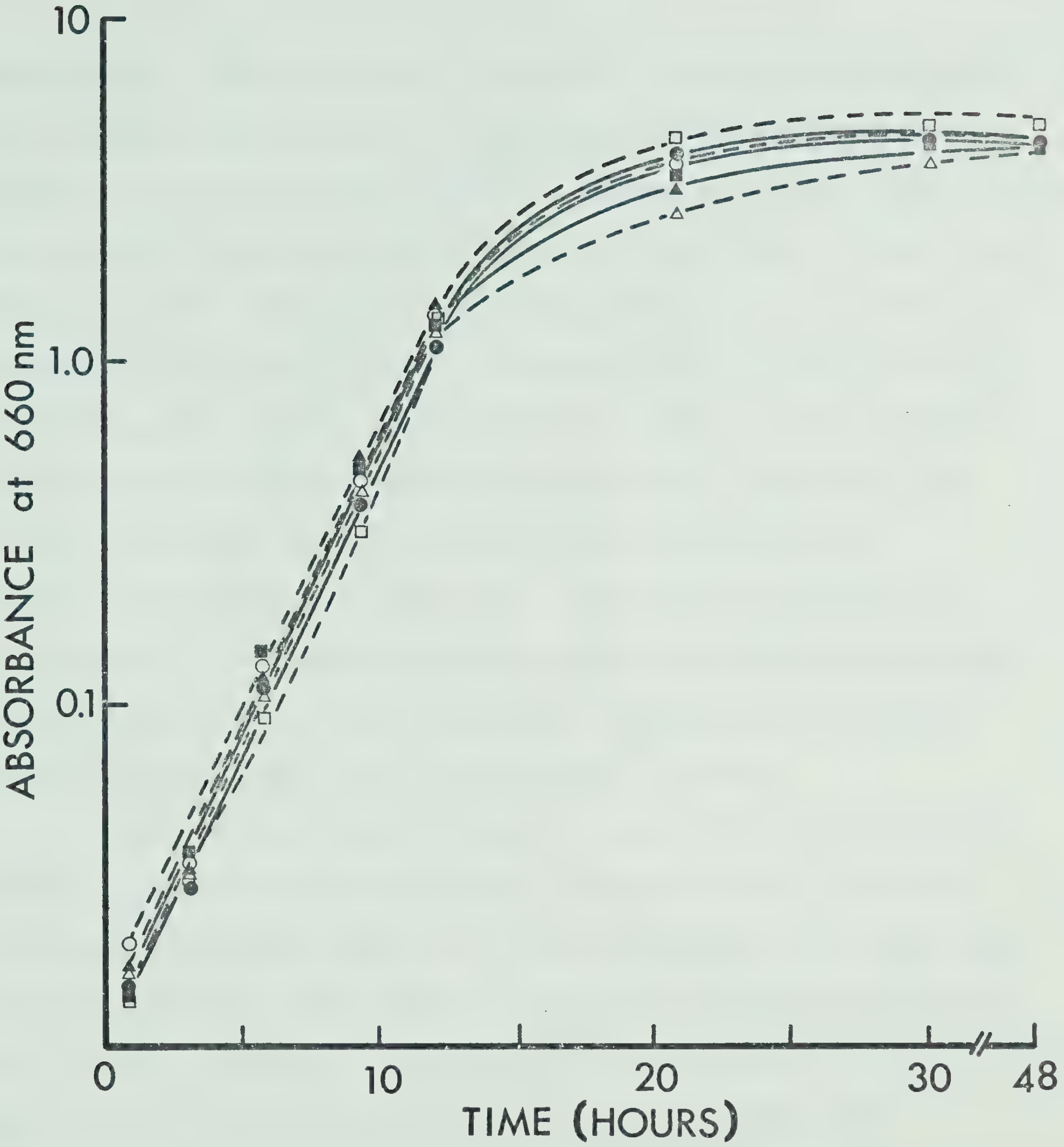

Fig. 8. Growth of Candida utilis on Basal Medium with Ammonium Sulphate as a Source of Nitrogen.

50 mg nitrogen/l ○— —○, 100 mg nitrogen/l ●—● ,

200 mg nitrogen/l ■—■ , 500 mg nitrogen/l △— —△ ,

1000 mg nitrogen/l ▲—▲ , 2500 mg nitrogen/l □— —□ .

concentration. Growth response of Candida to varying concentrations of urea produced results similar to those encountered using ammonium sulphate as a nitrogen source (Fig. 9). Increasing levels of urea produced only slight changes in growth during logarithmic and stationary phases of growth. Casein hydrolyzate was selected not only to be representative of complex source of nitrogen, but the presence of amino acids and peptide residues would resemble the enzymic digests of RSOM. Growth response to varying concentrations of casein hydrolyzate as a nitrogen source were similar to those results encountered using ammonium sulphate and urea (Fig. 10). Since growth of *Candida utilis* was found to be independent of nitrogen concentration during logarithmic and stationary growth periods, a nitrogen concentration of 1000 mg nitrogen/l was selected to be used throughout the study.

The results of Candida growth on basal medium using ammonium sulphate, urea and casein hydrolyzate (1000 mg nitrogen/l) as sources of nitrogen are shown in Fig. 11. It can be seen that casein hydrolyzate produces earlier and faster growth as compared to ammonium sulfate and urea, however, maximum cell growth was not achieved until 30 hours. Urea resulted in a more rapid growth rate than ammonium sulfate. Maximum cell yield using urea as the source of nitrogen was achieved after 18 hours while equivalent cell yield using ammonium sulphate was attained after 30 hours.

In addition to ammonium sulphate six inorganic sources of nitrogen were evaluated on their ability to support the growth of *C. utilis* (Fig. 12). Ammonium nitrate, potassium nitrate, sodium nitrate and ammonium phosphate all proved to be good sources of nitrogen.

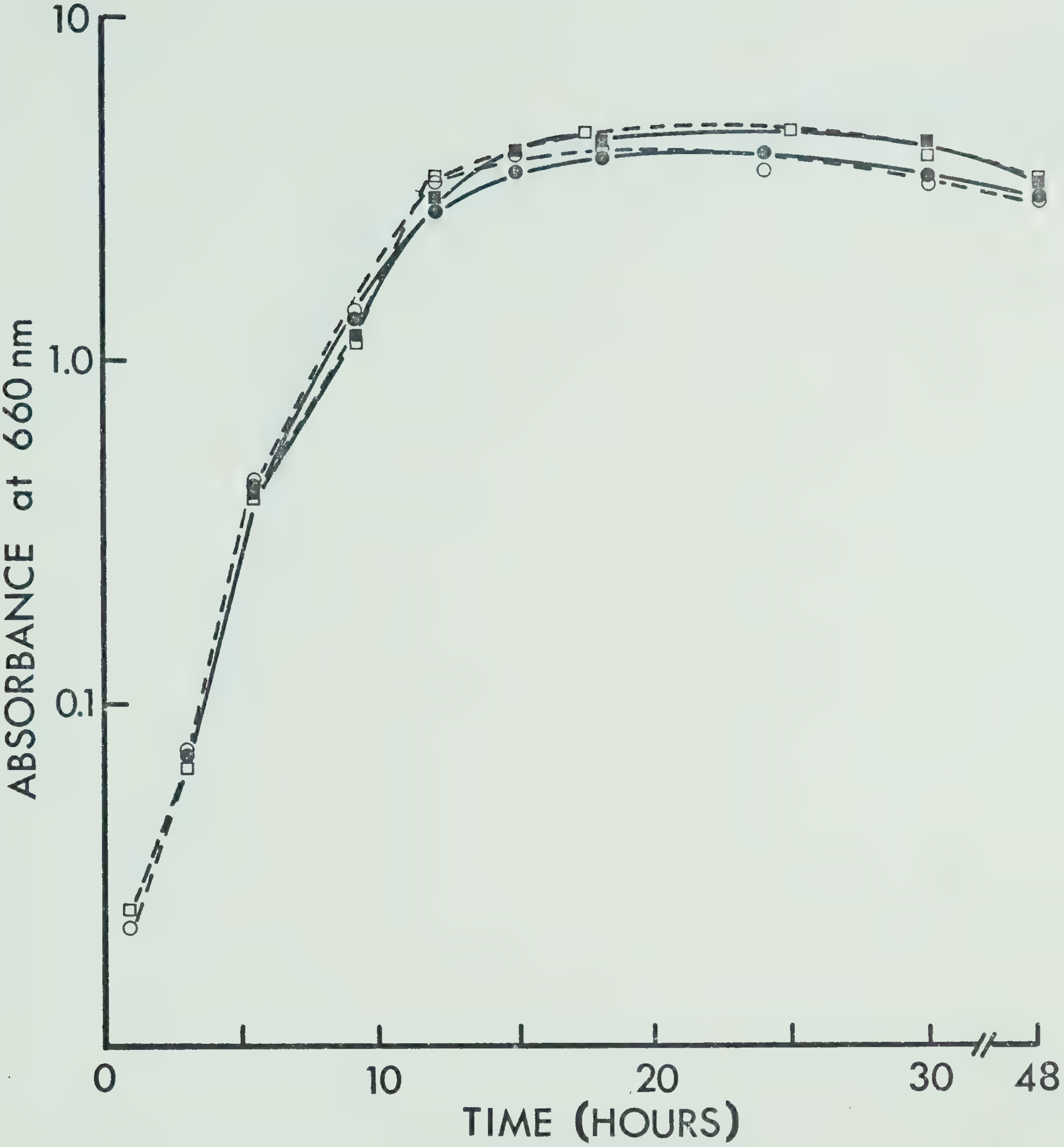

Fig. 9. Growth of <u>Candida</u> <u>utilis</u> on Basal Medium with Urea as a Source of Nitrogen.

50 mg nitrogen (urea)/l ○– –○ , 100 mg nitrogen/l ●—● ,

500 mg nitrogen/l ■—■ , 2500 mg nitrogen/l □– –□ .

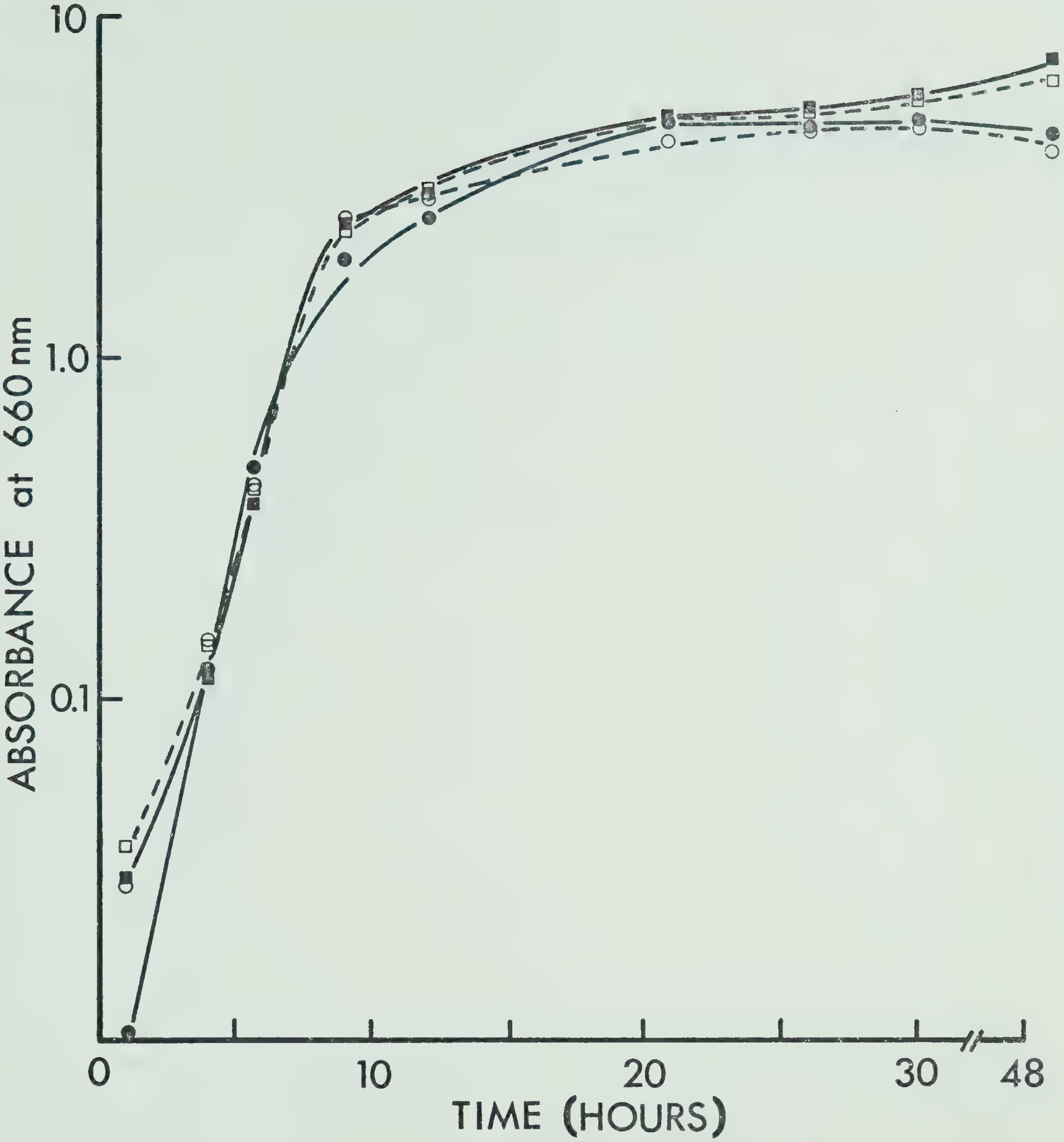

Fig. 10. Growth of <u>Candida utilis</u> on Basal Medium with Casein Hydrolyzate (Enzymic) as a Source of Nitrogen.

50 mg nitrogen/l ●—● , 100 mg nitrogen/l ○– –○ ,

1000 mg nitrogen/l □– –□ , 2500 mg nitrogen/l ■—■ .

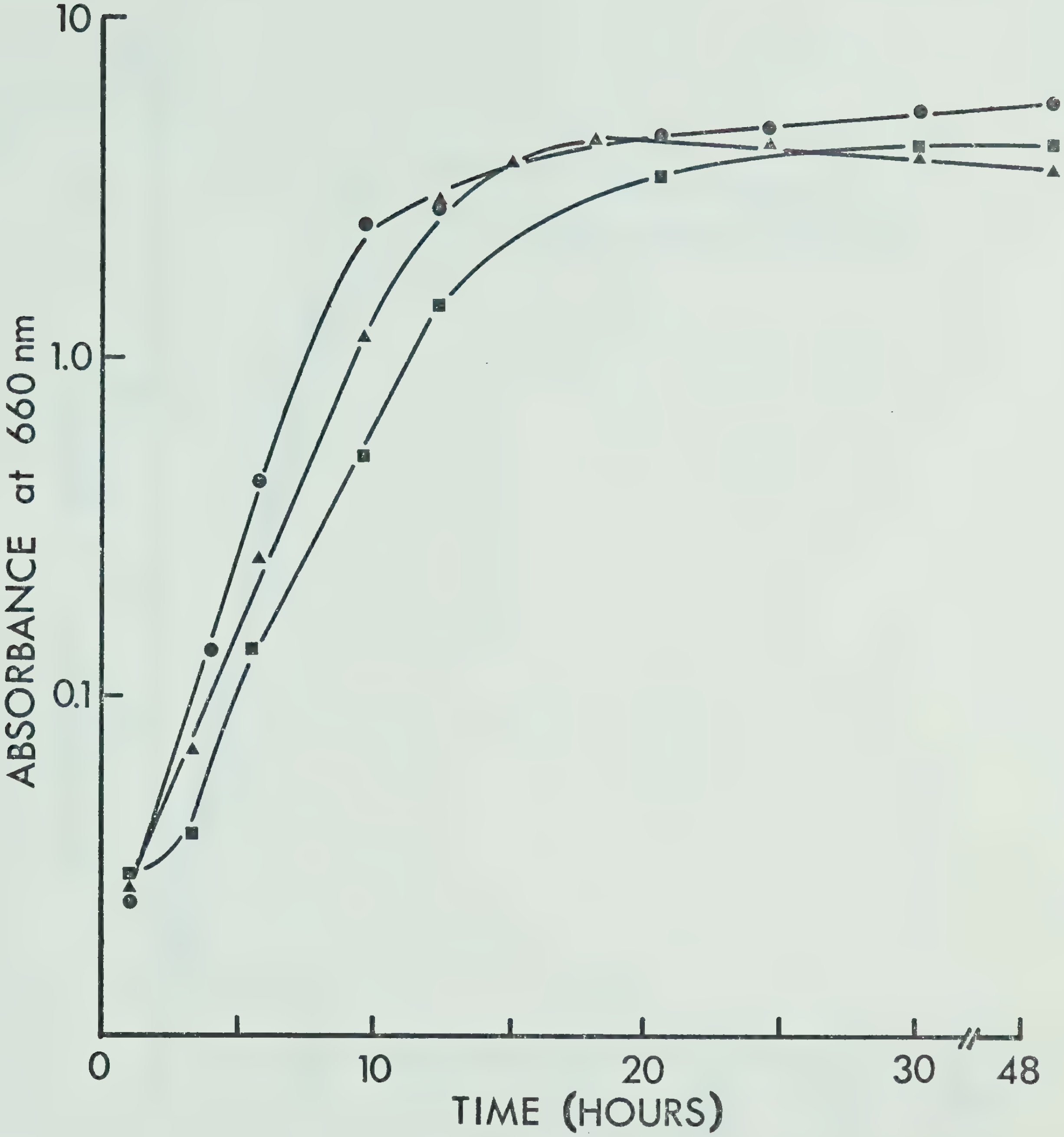

Fig. 11. Growth of <u>Candida utilis</u> on Basal Medium with Ammonium Sulphate, Urea and Casein Hydrolyzate (1000 mg nitrogen/l).

Ammonium sulphate ■—■ , Urea ▲—▲ ,

Casein hydrolyzate ●—● .

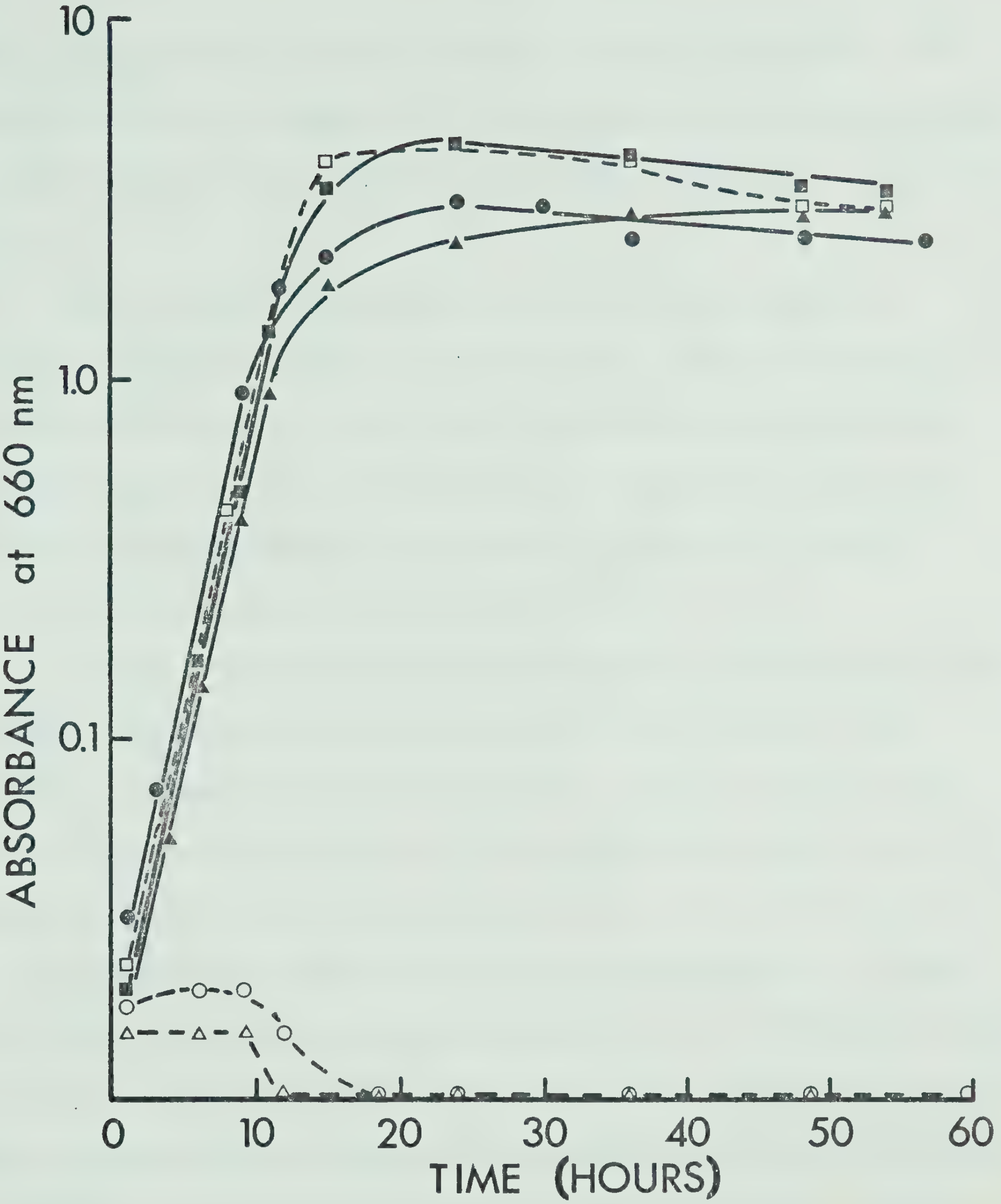

Fig. 12. Growth of <u>Candida utilis</u> on Inorganic Sources of Nitrogen (Basal Medium + 1000 mg nitrogen/l).

Ammonium nitrate ■——■ , Ammonium phosphate □– –□ ,

Potassium nitrate ●——● , Potassium nitrite ○– –○ ,

Sodium nitrate ▲——▲ , Sodium nitrite △– –△ .

However, both nitrites, not only failed to support growth but in fact appeared to be toxic. Changes in pH during growth of Candida utilis on the various inorganic sources of nitrogen were recorded and illustrated in Fig. 13.

Fig. 14 shows the ability of commercial and laboratory hydrolyzates or enzymic digests to serve as sole sources of energy, i.e., both carbon and nitrogen. Hydrochloric acid hydrolyzed rapeseed oil meal (RSOM-H) gave a cell yield comparable to soytone, (an enzymatic hydrolyzate of soybean meal), but appeared to be utilized slowly. Similar results were observed using RSOM-HA[30].

At this particular nitrogen concentration, medium formulation using pepsin and trypsin digested rapeseed oil meal provided some difficulty. Following sterilization, enzymic digests of RSOM formed particulate deposits resulting in a slurry-like mixture which made evaluation of growth spectrophotometrically impossible. In view of this, it was decided to reduce the nitrogen concentration of all rapeseed oil meal media preparations approximately ten-fold (110 mg nitrogen/1). With the exception of peptone and neopeptone, it is evident that all commercial peptones tested limited yeast growth after 10 hours and could be considered poor sources of nitrogen and carbon singularly. At the reduced concentrations (110 mg nitrogen/1), rapeseed oil meal preparations were shown to be superior to neopeptone as a source of energy but not as effective as soytone (Fig. 15). Pepsin digested rapeseed oil meal (RSOM-P) gave the slowest growth results in comparison to the other laboratory preparations.

Fig. 16 represents the growth of Candida on two commercial media,

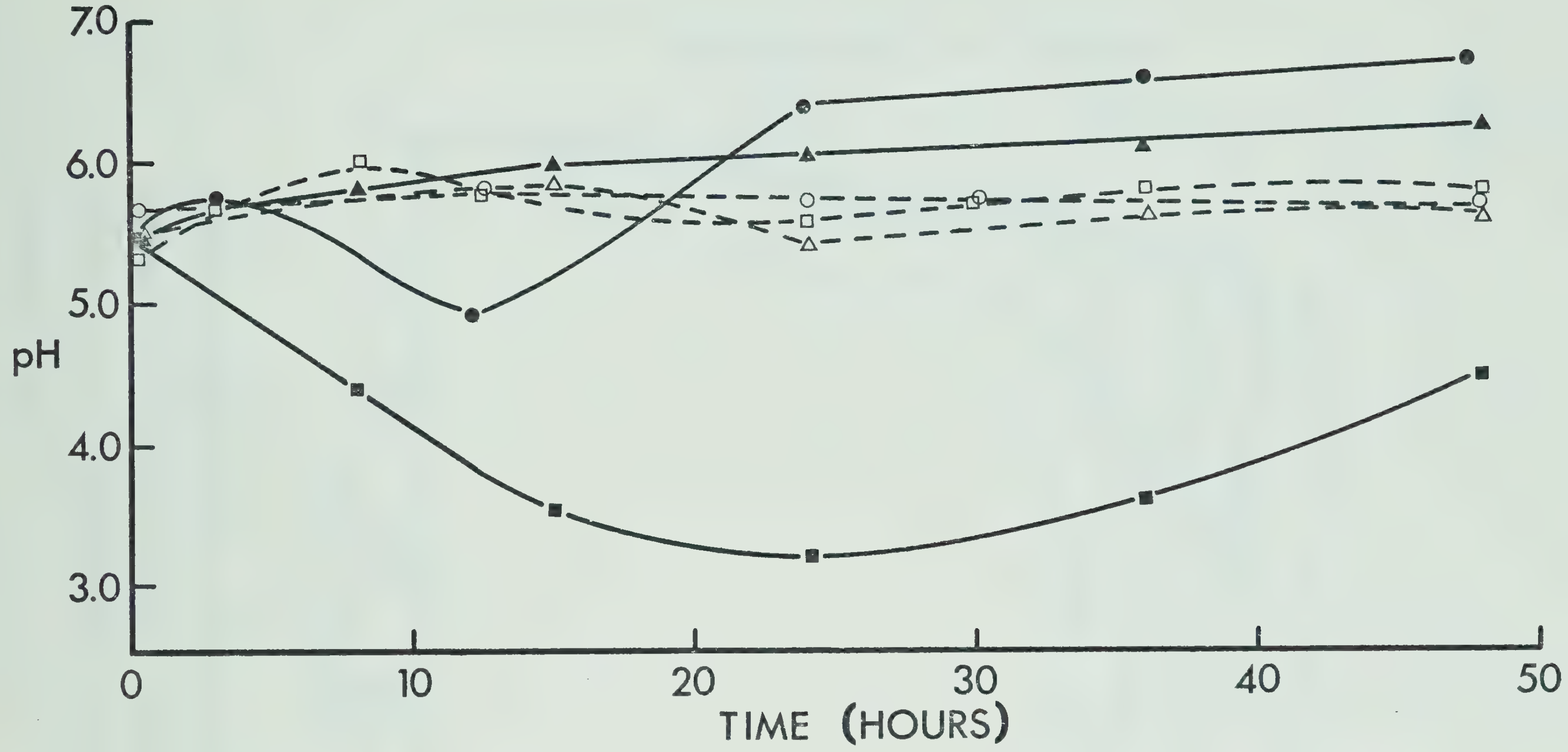

Fig. 13. Changes in pH During Growth of Candida utilis on Inorganic Sources of Nitrogen (Basal Medium + 1000 mg nitrogen/l).

Ammonium phosphate □– –□ , Ammonium nitrate ■—■ ,

Potassium nitrate ●—● , Potassium nitrite ○– –○ ,

Sodium nitrate ▲—▲ , Sodium nitrite △– –△.

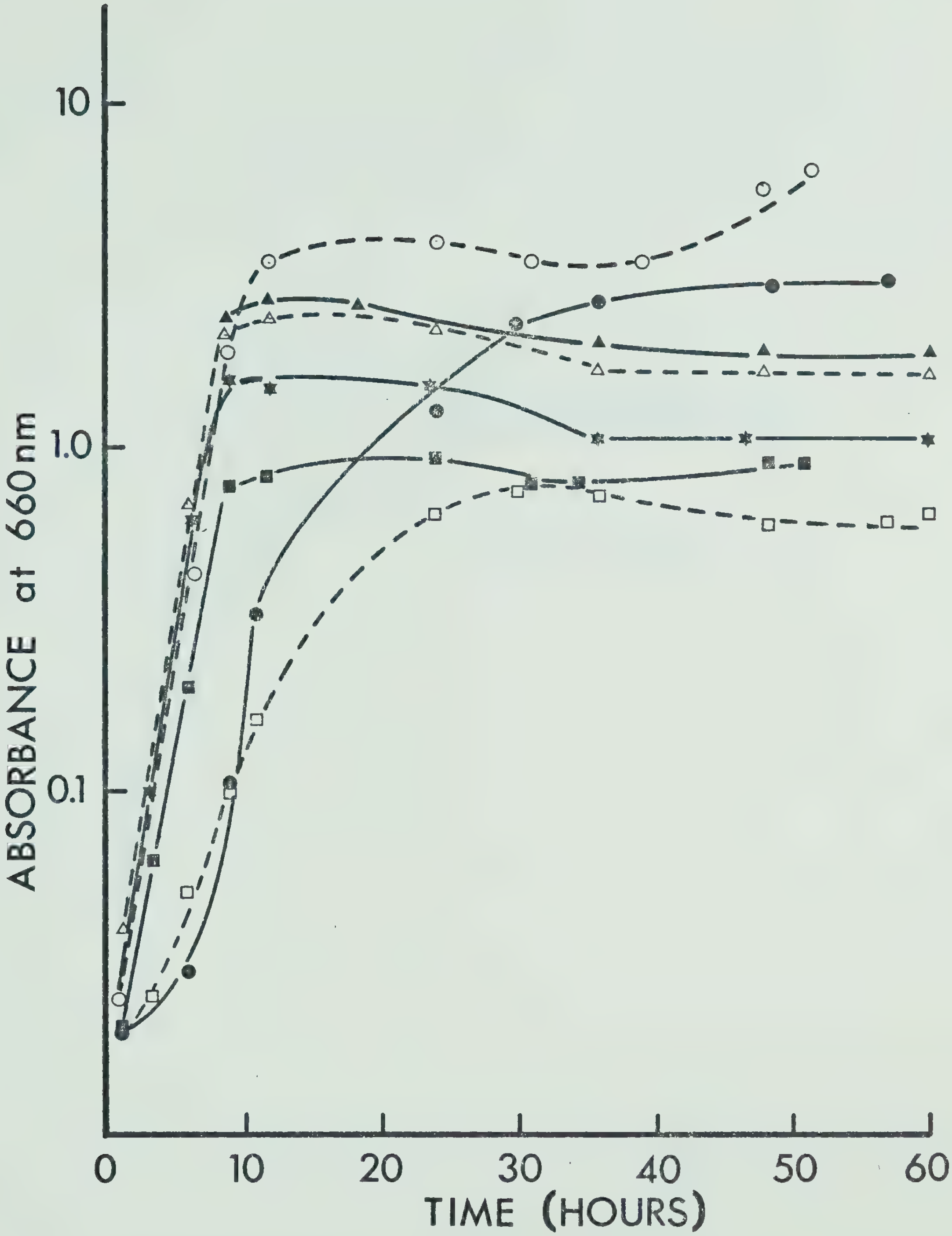

Fig. 14. Growth of Candida utilis on Protein Digests (1000 mg nitrogen/l).

Soytone ○– –○ , RSOM-H ●—● , Tryptone ▲—▲ ,

Tryptose △– –△ , Proteose-Peptone ✶—✶ , Peptone ■—■ ,

Neopeptone □—□ .

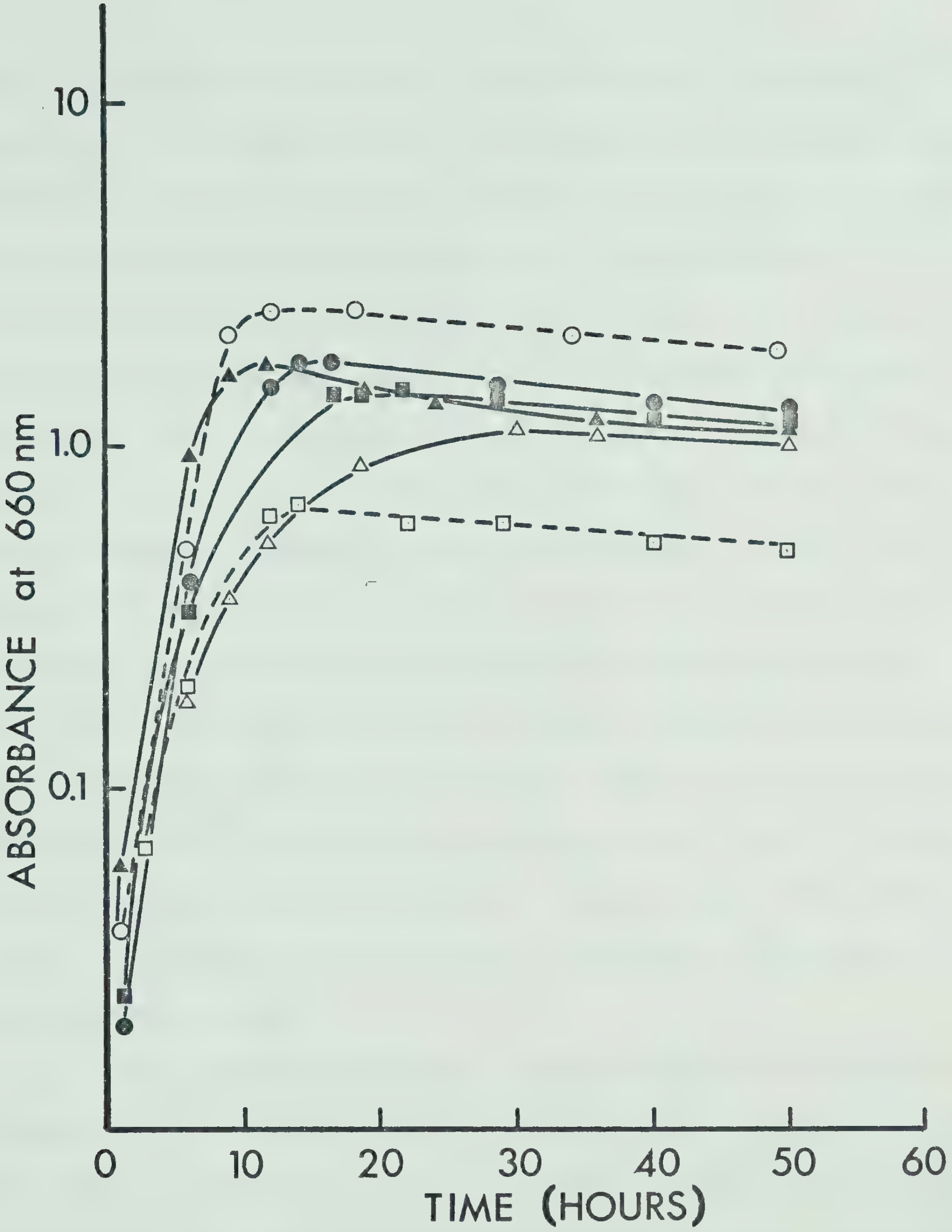

Fig. 15. Growth of <u>Candida utilis</u> on Rapeseed Oil Meal Preparations, Soytone and Neopeptone (110 mg nitrogen/l).

Soytone ○— —○ , RSOM-H ●—● , RSOM-HA^{30} ■—■ ,

RSOM-P △—△ , RSOM-T ▲—▲ , Neopeptone □— —□.

namely, Sabouraud Dextrose Broth (1433 mg nitrogen (neopeptone)/1 + 20 g dextrose/1) and Mycological Broth (1000 mg nitrogen (soytone)/1 + 40 g dextrose/1). Due to difficulties incurred in formulation of rapeseed oil meal media at high nitrogen concentration (1000 mg nitrogen/1) only acid hydrolyzed rapeseed oil meal (RSOM-H) was evaluated in its ability to replace the nitrogen requirements found in Sabouraud Dextrose Broth and Mycological Broth. Equivalent dextrose concentration was maintained. It can be seen that the simulated commercial media resulted in poor growth respense as compared to the original commercial media. Both Sabouraud Dextrose Broth and Mycological Broth gave early and rapid growth when compared to their rapeseed oil meal formulated counterparts. Using RSOM-H as a nitrogen source at 1000 mg nitrogen/1 incorporated with basal medium produced growth results better than the simulated commercial media but was inferior to the commercial media. The basal medium contained only 2 g dextrose/1. It would appear that growth response is related to the nitrogenous components of the media and not to the dextrose content.

The suitability of acid or enzymic digests of rapeseed oil meal as components of mycological media is represented in Figs. 17 - 21. At low nitrogen concentration, 110 mg nitrogen/1, RSOM-H gave good growth providing dextrose was added at either 20 g dextrose/1 or 40 g dextrose/1 (Fig. 17). RSOM-H supplemented with 40 g dextrose/1 gave better growth than Sabouraud Dextrose Broth but lower dextrose concentration (20 g dextrose/1) resulted in less growth. Dextrose supplementation was shown to aid growth and produced between 3 - 6 fold increases in growth as compared to RSOM-H used alone.

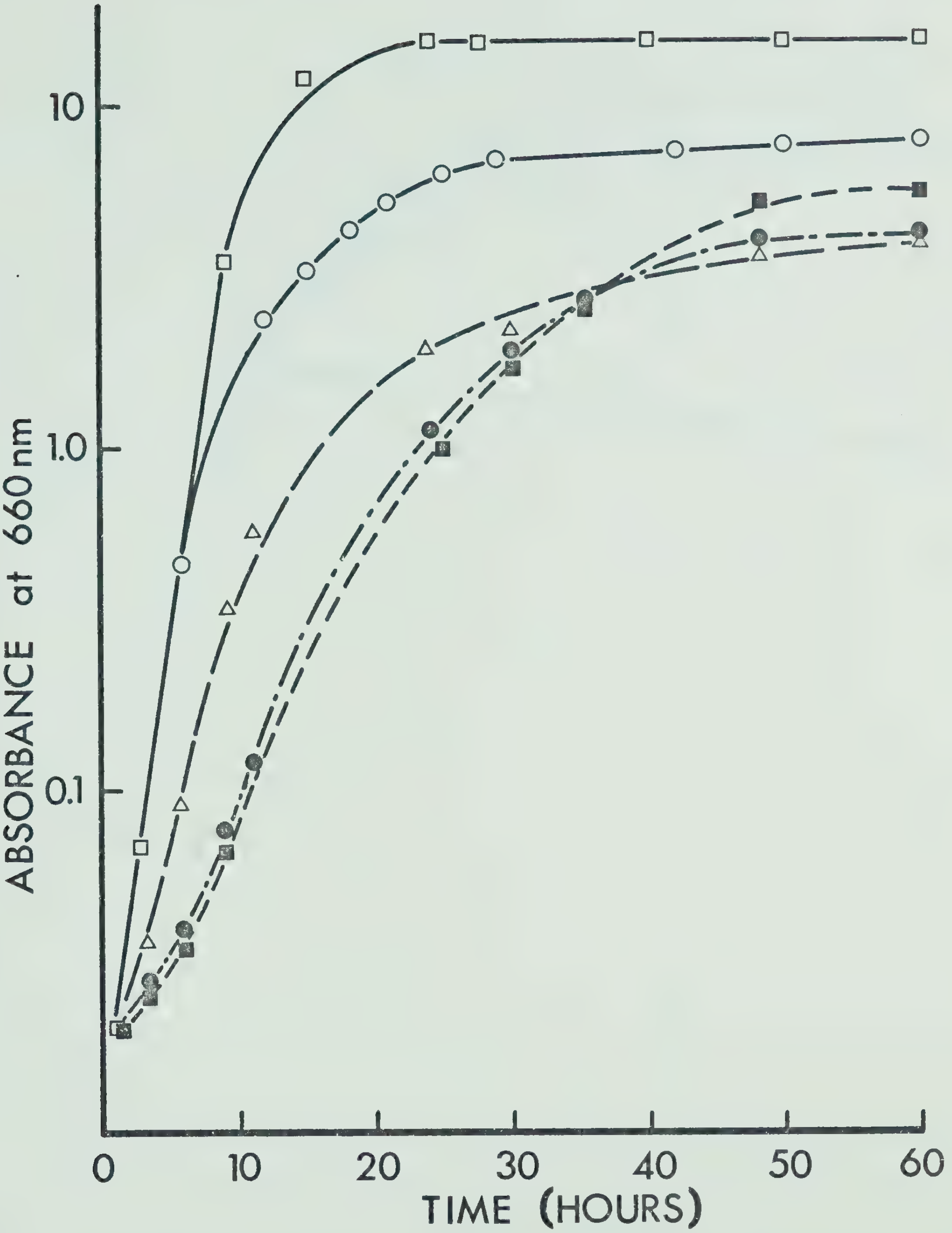

Fig. 16. Growth of <u>Candida</u> <u>utilis</u> on Mycological Broth, Sabouraud Dextrose Broth, RSOM-H Equivalents and Basal Medium. MB □—□ , SDB ○——○ , RSOM-H (MB eq.) ■— —■ , RSOM-H (SDB eq.) ●—·—●, Basal Medium + 1000 mg nitrogen (RSOM-H)/1 △— —△ .

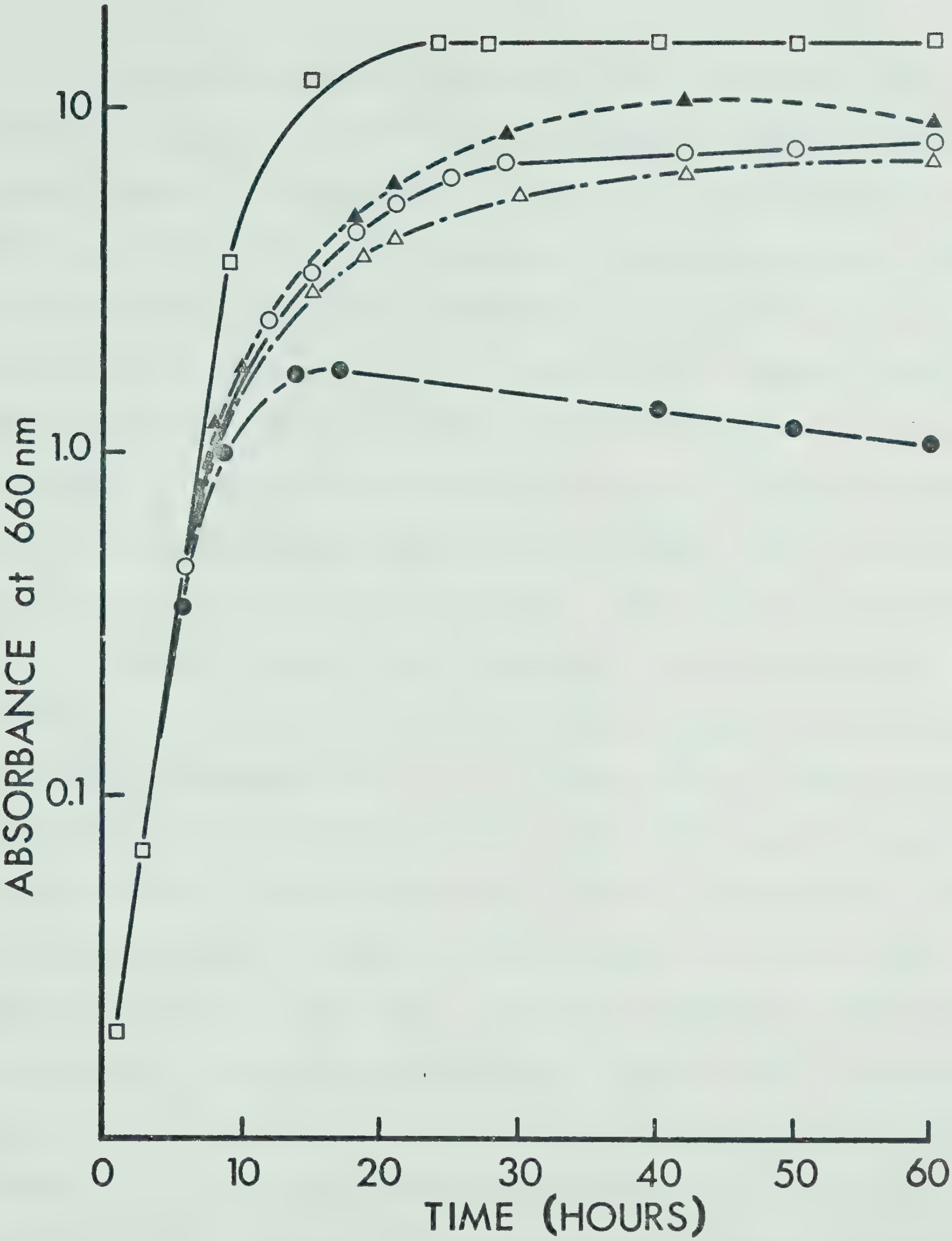

Fig. 17. Growth of <u>Candida utilis</u> on Mycological Broth, Sabouraud Dextrose Broth; and RSOM-H (110 mg nitrogen/l) With and Without Dextrose Supplementation.

MB □—□ , SDB ○—○ , RSOM-H ●— —● , RSOM-H + 20 g dextrose/l △—•—△ , RSOM-H + 40 g dextrose/l ▲— —▲ .

Growth of Candida on rapeseed oil meal hydrolyzed by the "autoclave" procedure (RSOM-HA^{30}) is illustrated in Fig. 18. In contrast to data obtained using RSOM-H as a source of nitrogen, addition of dextrose to RSOM-HA^{30} at both 20 and 40 g/l supported comparable growth. Both preparations gave results comparable to those obtained with Sabouraud Dextrose Broth. However, addition of dextrose did increase RSOM-HA^{30} utilization 7 - 8 times as compared to using RSOM-HA^{30} alone. It is interesting to note that in both studies using acid hydrolyzed RSOM, Mycological Broth gave the highest growth response. RSOM-H and RSOM-HA^{30}, even with addition of equivalent dextrose, were inferior to this medium.

Enzymatic digestion of rapeseed oil meal under alkaline conditions, i.e., trypsin digestion, produced a digest which gave growth responses as presented in Fig. 19. As shown earlier, dextrose supplementation produced cell yields more favorable than using trypsin digested rapeseed oil meal (RSOM-T) singularly. Dextrose supplementation produced 4 - 5 fold increases in growth. After 24 hours, the tryptic digest media containing 20 and 40 g dextrose/l gave growth comparable to Sabouraud Dextrose Broth. At no time throughout the study was RSOM-T formulated media able to support growth levels equivalent to Mycological Broth, however, it is noteworthy to mention that RSOM-T media did produce earlier growth than either Sabouraud Dextrose Broth and Mycological Broth during the initial 8 hours.

Growth response of Candida on pepsin digested rapeseed oil meal (RSOM-P) is shown in Fig. 20. Pepsin digested rapeseed oil meal is representative of an acidic, enzymic peptone. At low nitrogen concentration (110 mg nitrogen/l) RSOM-P with or without added dextrose was shown

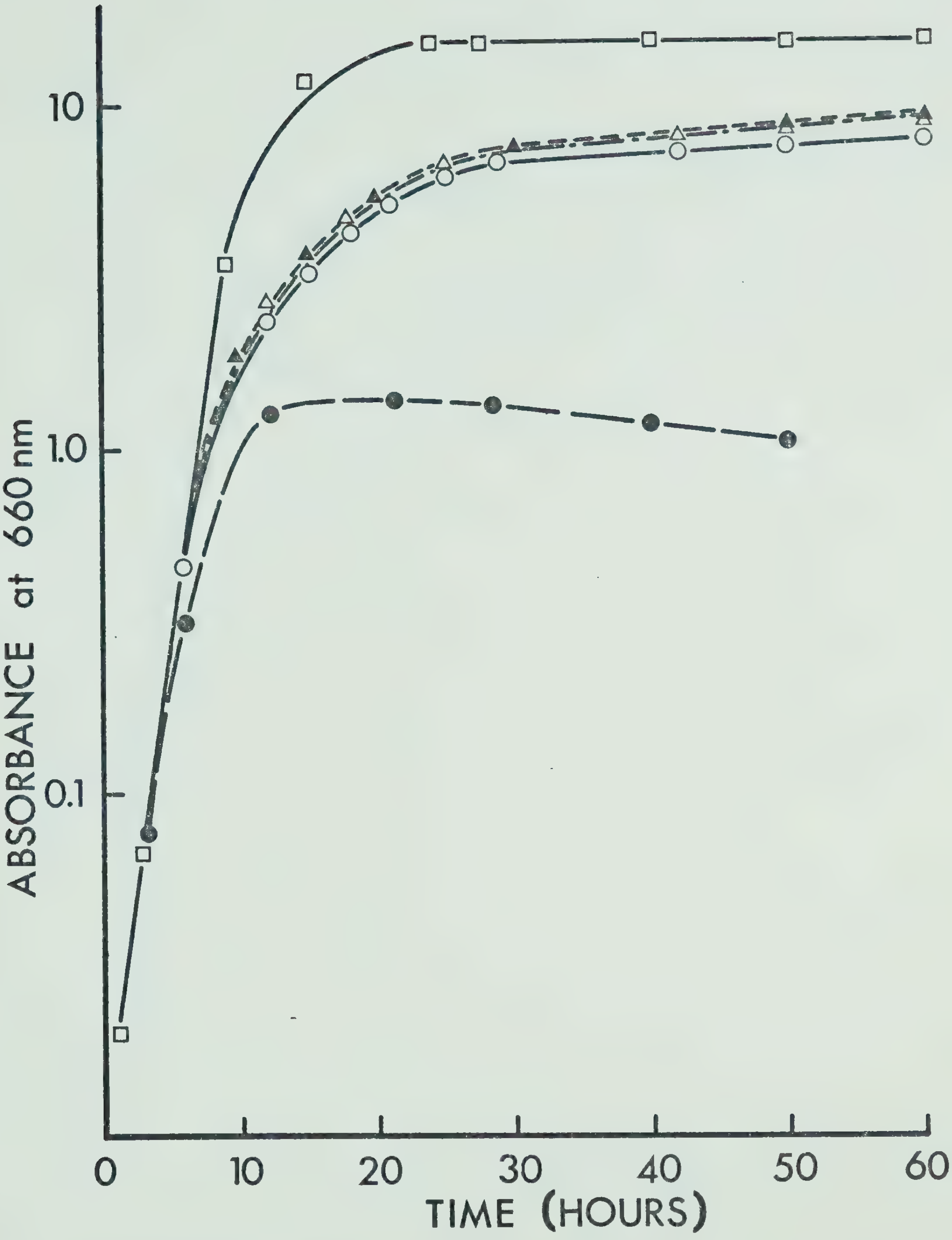

Fig. 18. Growth of Candida utilis on Mycological Broth, Sabouraud Dextrose Broth; and RSOM-HA^{30} (110 mg nitrogen/l) With and Without Dextrose Supplementation.

MB □—□ , SDB ○—○ , RSOM-HA^{30} ●— —● , RSOM-HA^{30} + 20 g dextrose/l △—•—△ , RSOM-HA^{30} + 40 g dextrose/l ▲— —▲ .

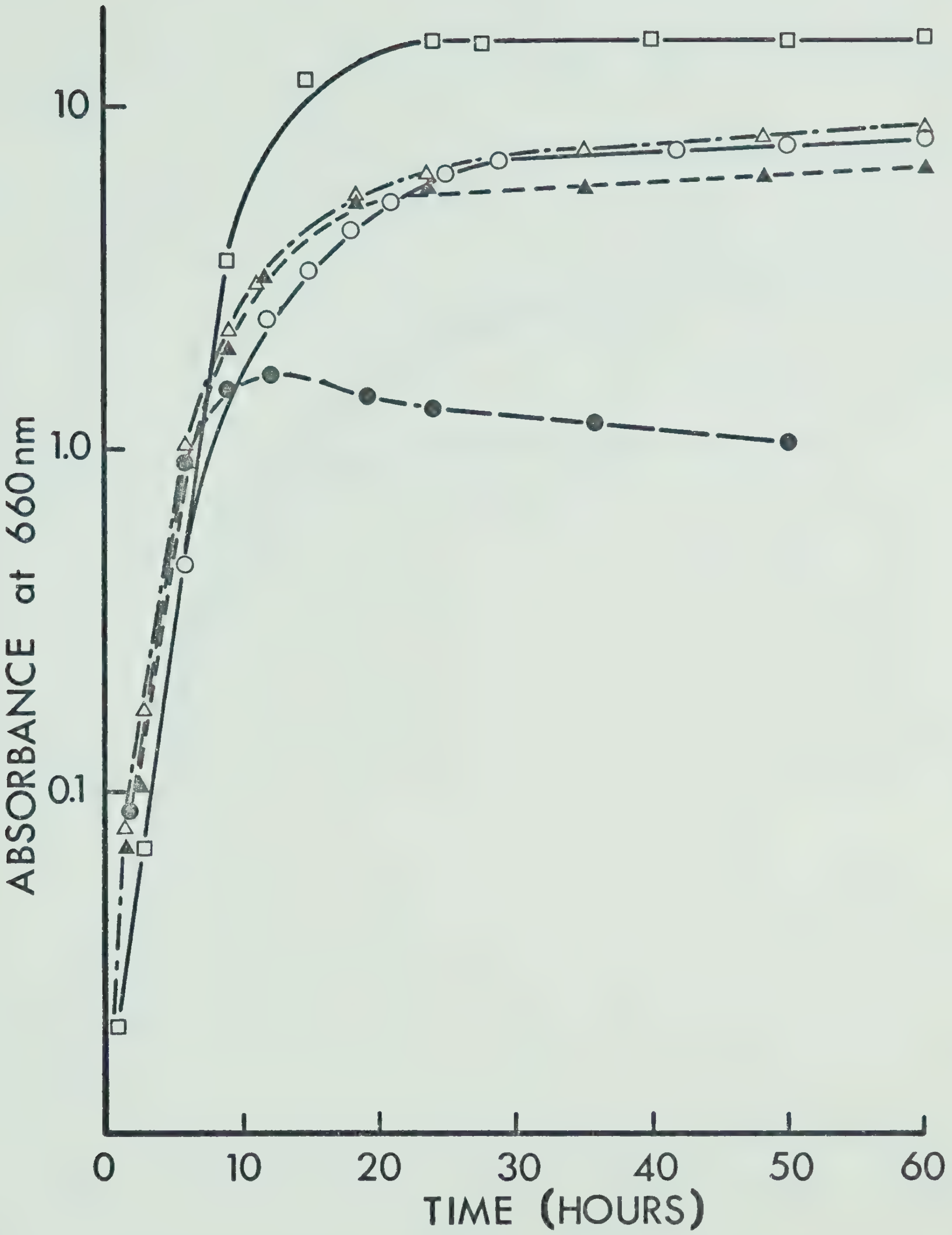

Fig. 19. Growth of <u>Candida utilis</u> on Mycological Broth, Sabouraud Dextrose Broth; and RSOM-Tryptic Digests (110 mg nitrogen/l) With and Without Dextrose Supplementation.

MB □—□ , SDB ○—○ , RSOM-T ●— —● , RSOM-T + 20 g dextrose/l △—•—△ , RSOM-T + 40 g dextrose/l ▲— —▲ .

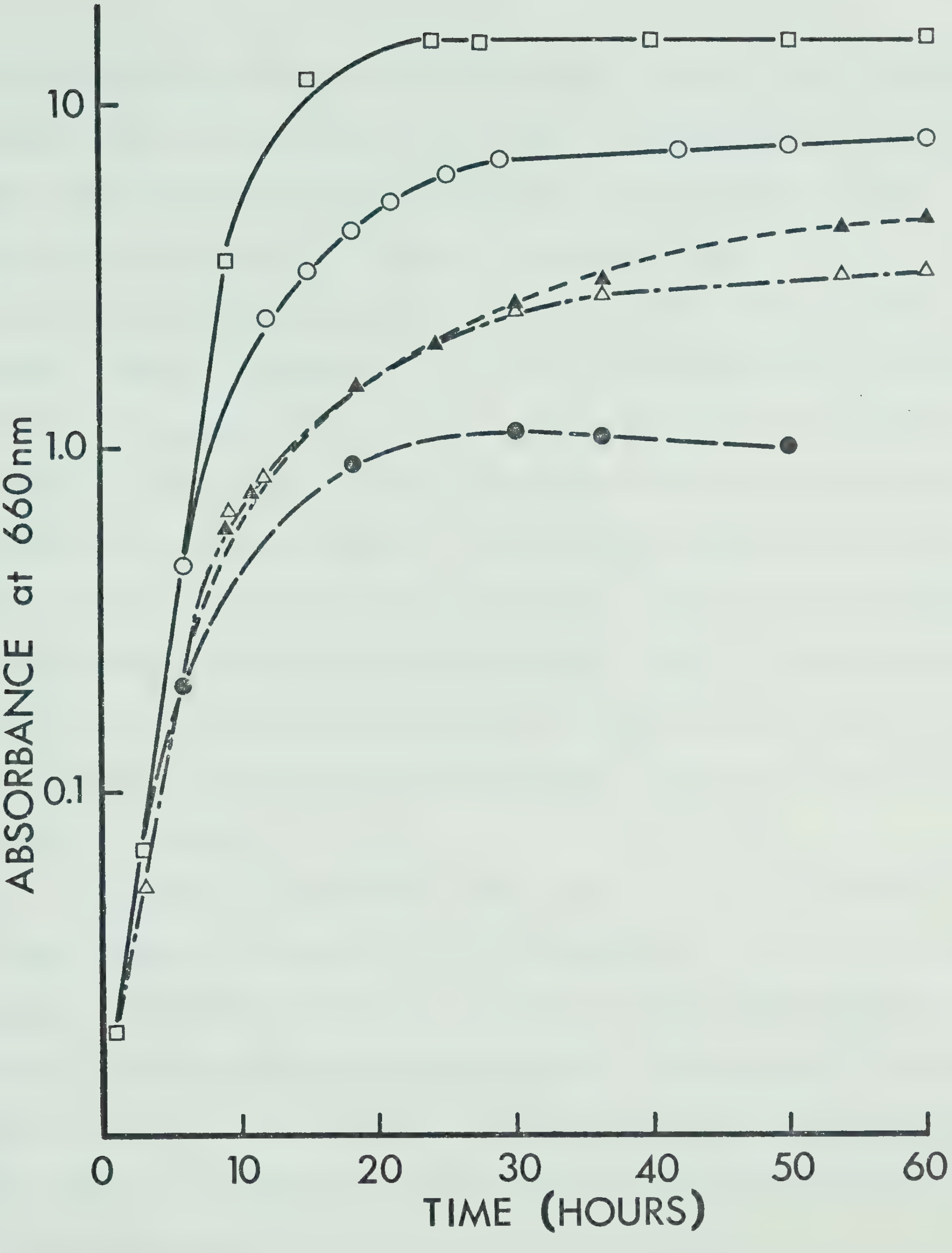

Fig. 20. Growth of <u>Candida utilis</u> on Mycological Broth, Sabouraud Dextrose Broth; and RSOM-Peptic Digests (110 mg nitrogen/l) With and Without Dextrose Supplementation.

MB □——□ , SDB ○——○ , RSOM-P ●— —●, RSOM-P + 20 g dextrose/l △—•—△, RSOM-P + 40 g dextrose/l ▲— —▲.

to promote less growth than commercial media. The cell yield on Sabouraud Dextrose Broth and Mycological Broth was 2 - 4 times that attained using RSOM-P and equivalent dextrose. The addition of dextrose to RSOM-P increased yeast yield 2 - 4 times as compared to RSOM-P alone. Fig. 21 represents the growth response of Candida to RSOM-P with and without dextrose when the nitrogen content of the formulated media was increased to 500 mg nitrogen/l. This nitrogen concentration was the maximum quantity of RSOM-P which could be formulated without the formation of interferring extraneous material. The addition of dextrose to pepsin digested rapeseed oil meal resulted in growth yields better than Sabouraud Dextrose Broth but not as good as Mycological Broth. Increased dextrose supplementation produced no difference in Candida growth. These results were similar to those reported using RSOM-HA[30] (110 mg nitrogen/l) as a source of nitrogen.

Soytone was repeatedly shown to be a superior source of nitrogen when used singularly or when commercially formulated as a media component of Mycological Broth. Fig. 22 represents the utilization of soytone at reduced nitrogen concentrations (110 mg nitrogen/l) with and without dextrose. It is evident that soytone (110 mg nitrogen/l) with added dextrose is comparable to Sabouraud Dextrose Broth (1433 mg nitrogen/l, 20 g dextrose/l).

In addition to Sabouraud Dextrose Broth and Mycological Broth the suitability of two other commercially available media to promote growth were evaluated. Czapek-Dox Broth is a defined liquid medium, nearly neutral in reaction, designed for the cultivation of fungi and bacteria capable of utilizing inorganic nitrogen. Sodium nitrate is the

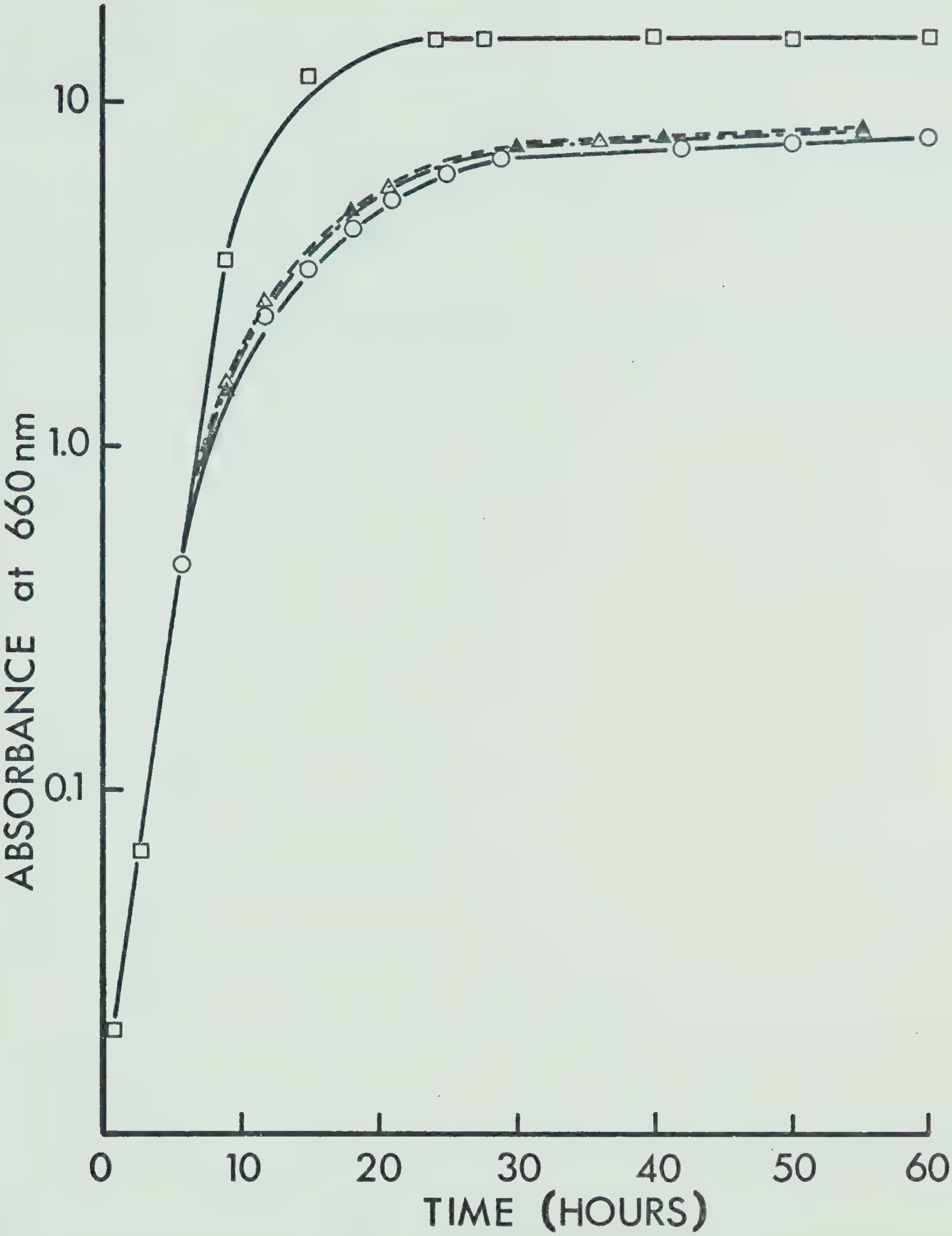

Fig. 21. Growth of <u>Candida</u> <u>utilis</u> on Mycological Broth, Sabouraud Dextrose Broth; and RSOM-Peptic Digests (500 mg nitrogen/l) With and Without Dextrose Supplementation.

MB □—□ , SDB ○—○ , RSOM-P + 20 g dextrose/l △—•—△,

RSOM-P + 40 g dextrose/l ▲—▲ .

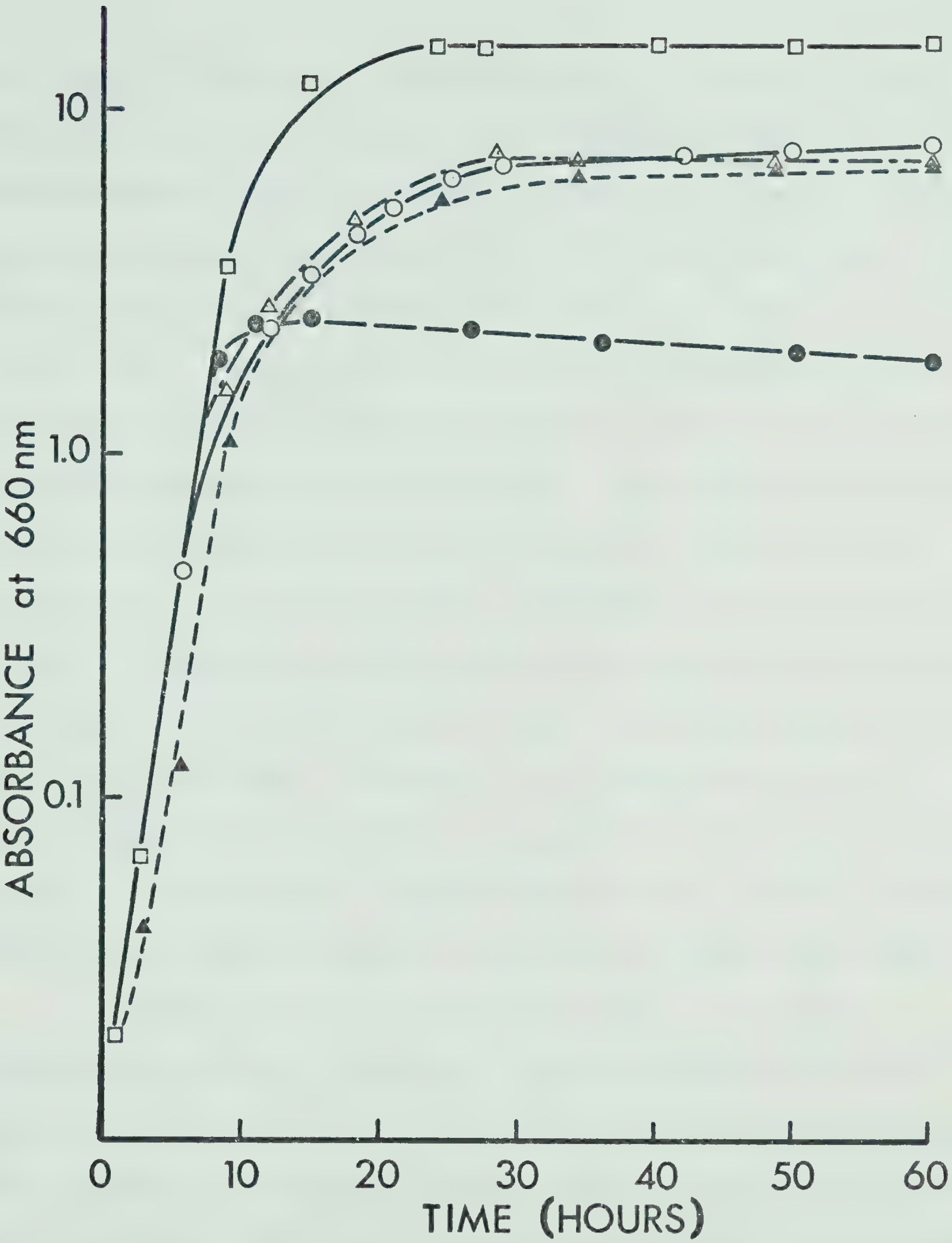

Fig. 22. Growth of <u>Candida utilis</u> on Mycological Broth, Sabouraud Dextrose Broth; and Soytone (110 mg nitrogen/l) With and Without Dextrose Supplementation.

MB □——□ , SDB ○——○ , Soytone ●— —●, Soytone + 20 g dextrose/l △—•—△, Soytone + 40 g dextrose/l ▲— —▲.

sole source of nitrogen and saccharose serves as a source of carbon in this medium. As illustrated in Fig. 23, Czapek-Dox Broth is superior to Sabouraud Dextrose Broth but inferior to Mycological Broth. Growth of *Candida utilis* on this particular medium is slow, nevertheless, a high yield of yeast cells is attained after 36 hours as compared to Sabouraud Dextrose Broth. Tryptic Soy Broth (pH 7.30) is an undefined medium composed of tryptone, a pancreatic digest of casein, soytone, dextrose, dipotassium phosphate and sodium chloride. This medium produced growth comparable to Mycological Broth only during the initial 9 hours of growth (logarithmic phase of growth), thereafter growth response decreased although a higher cell yield than Sabouraud Dextrose Broth was attained. In comparison to Czapek-Dox medium, Tryptic Soy Broth produced earlier and faster growth rates. Rapeseed oil meal preparations generally provided growth responses earlier to Czapek-Dox and intermediate to Tryptic Soy Broth; however, both TSB and Czapek-Dox produced slightly superior cell yields as compared to rapeseed oil meal preparations.

Normally, the pH of Mycological Broth is 7.0. Difco Laboratories report that "generally, acidic conditions produce earlier growth but under alkaline conditions greater growth yields are reported". This statement is substantiated using Mycological Broth under alkaline and acidic conditions (Fig. 23). Equivalent growth yields are achieved after 24 hours, but after 60 hours cell yield on Mycological Broth (pH 7.0) is increased by 5 absorbance units (1.2 mg cells/ml). Acidic conditions promoted early yeast growth. Consequently, the effect of alkaline cultural conditions on rapeseed oil meal media was investigated (Figs. 24 - 25). Mycological Broth (pH 7.0) proved to be superior to the

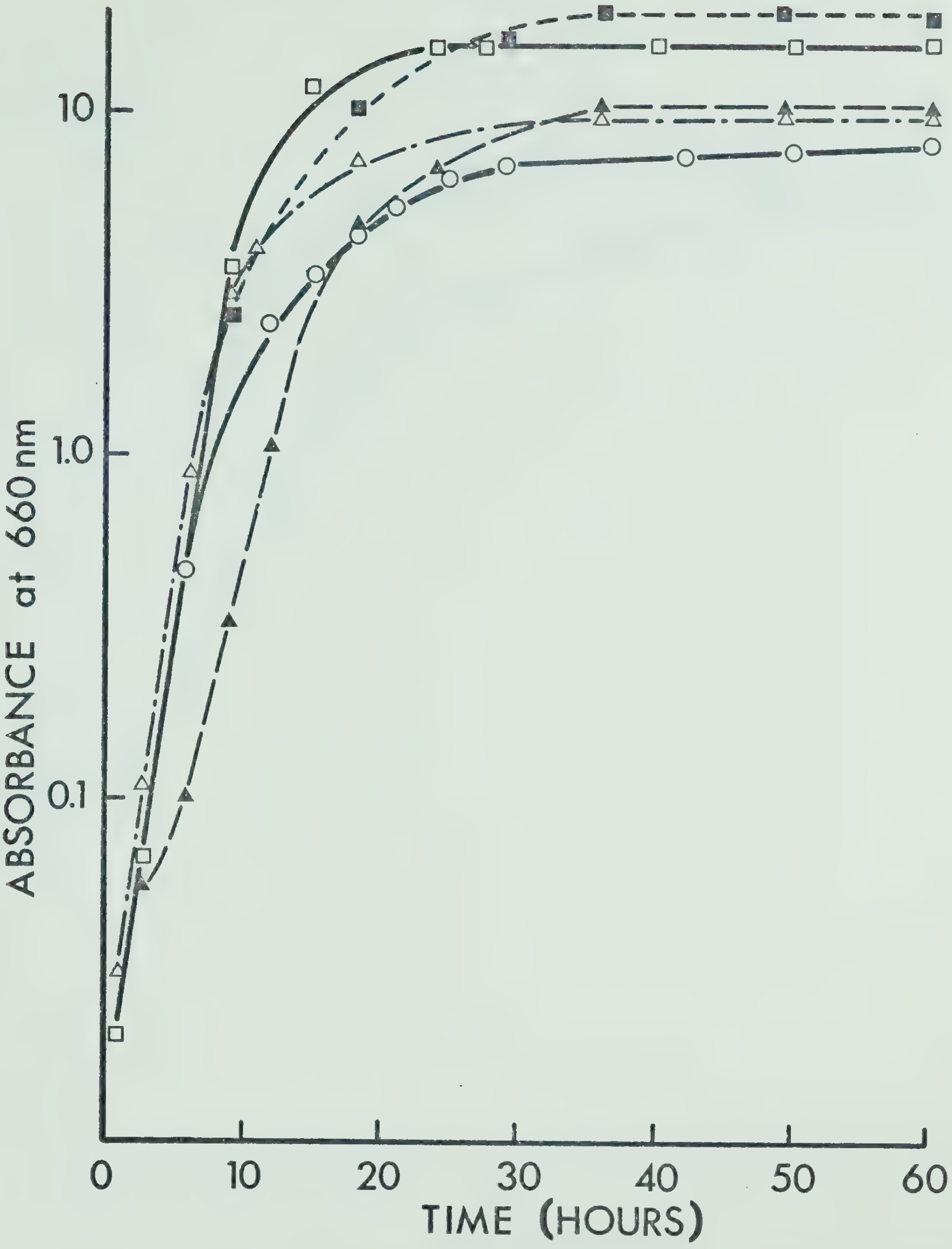

Fig. 23. Growth of Candida utilis on Selected Commercial Media.
MB (pH 5.5) □—□ , MB (pH 7.0) ■— —■ , SDB ○—○ ,
TSB △—•—△, Czapek-Dox ▲— —▲ .

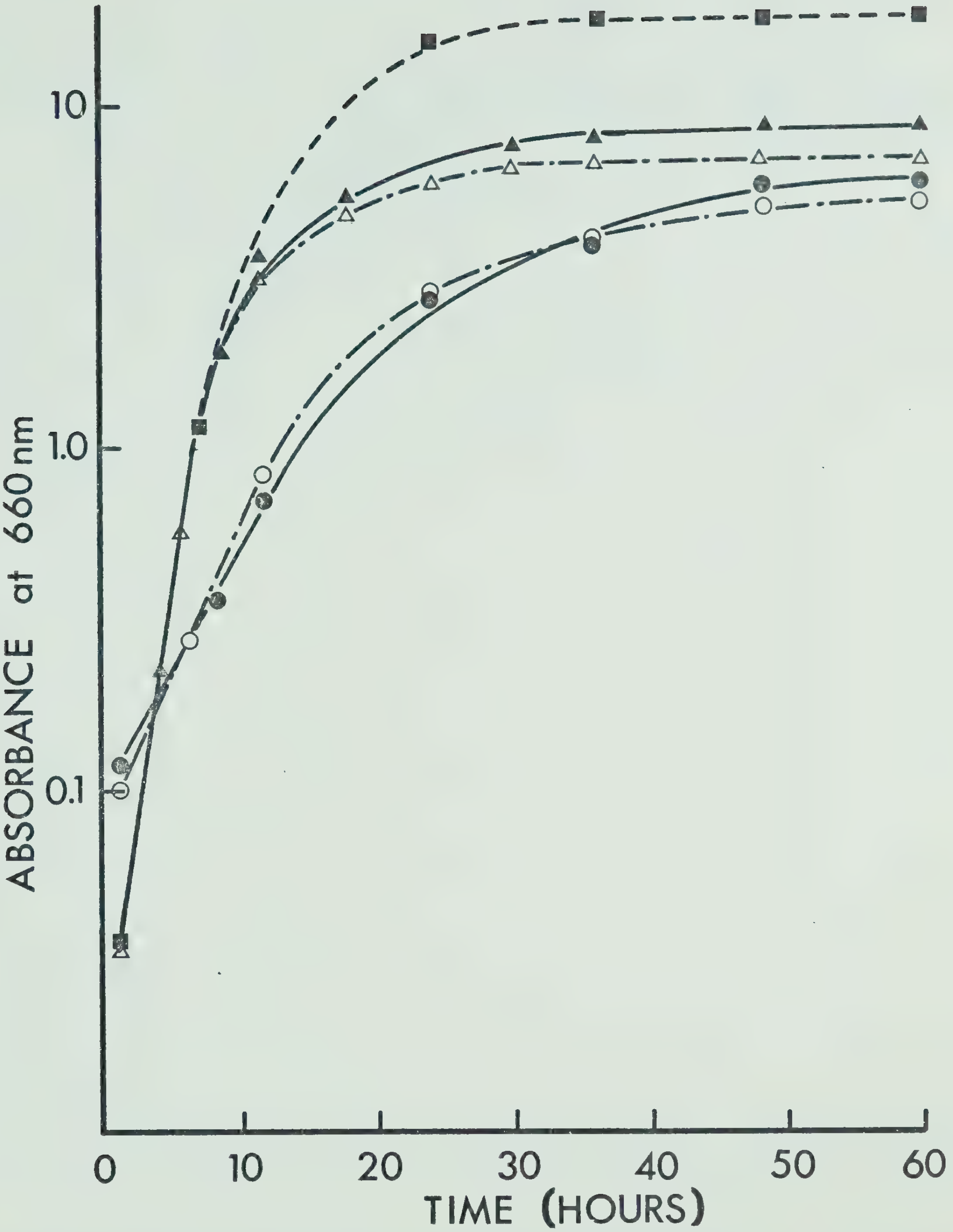

Fig. 24. Growth of Candida utilis on Mycological Broth and RSOM-Enzymic Digests (110 mg nitrogen/l) With Dextrose Supplementation at pH 7.0.

MB (pH 7.0) ■— —■ , RSOM-T + 20 g dextrose/l △—•—△ , RSOM-T + 40 g dextrose/l ▲——▲ , RSOM-P + 20 g dextrose/l ○—•—○, RSOM-P + 40 g dextrose/l ●——● .

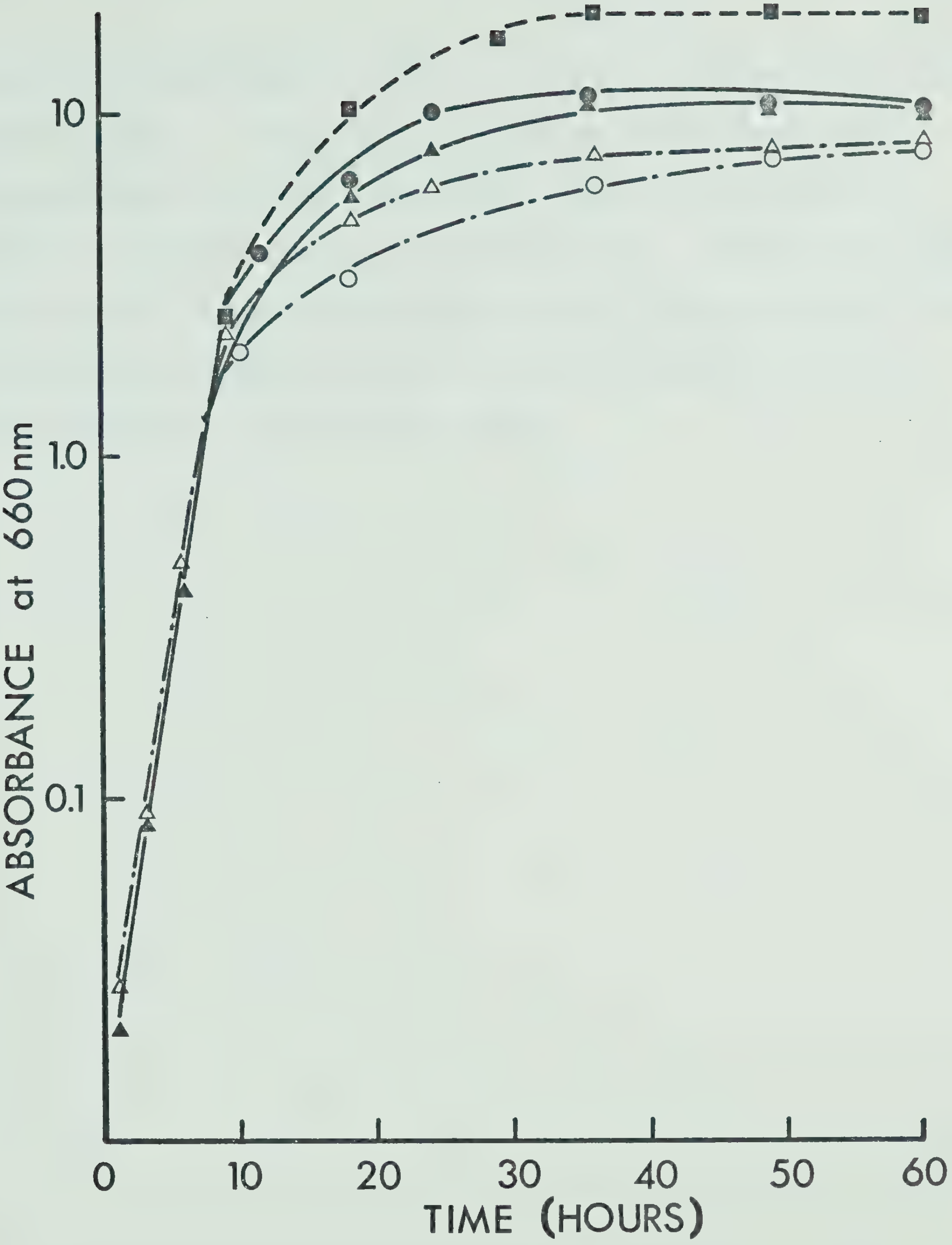

Fig. 25. Growth of <u>Candida</u> <u>utilis</u> on Mycological Broth and RSOM-Acid Digests (110 mg nitrogen/l) With Dextrose Supplementation at pH 7.0.

MB (pH 7.0) ■- - -■ , RSOM-H + 20 g dextrose/l ○—·—○ , RSOM-H + 40 g dextrose/l ●——● , RSOM-HA^{30} + 20 g dextrose/l △—·—△ , RSOM-HA^{30} + 40 g dextrose/l ▲——▲ .

rapeseed oil meal formulated media. However, it is interesting to note that the effect of increasing dextrose concentration became more pronounced than in previous experiments. Higher concentrations of dextrose invariably produced greater cell yields. After 60 hours, the media at pH 7.0, with the exception of RSOM-T (110 mg nitrogen/l) + 20 g dextrose/l, gave higher cell yields than did identical media under acidic conditions in previous experiments.

DISCUSSION AND CONCLUSION

The nutritional needs of microorganisms are diverse, because they differ inherently in ability to synthesize essential cellular constituents from simple nutrients. Autotrophs, for example, are able to grow using either the energy liberated by the oxidation of certain inorganic compounds, or the energy from light. The former are chemosynthetic autotrophs and the latter are photosynthetic autotrophs. The nutrient requirements of these organisms are relatively simple (inorganic ions, simple salts, inorganic nitrogen, carbon dioxide, etc.). Heterotrophs or chemo-organothrophs require more complex organic compounds. Fungi, most protozoa, and many bacteria are heterotrophs. The complexity of their nutrient requirements vary widely according to species or even a particular strain.

The wide range of nutritional requirements of microorganisms is reflected in the large number of media developed for their cultivation and isolation. Although numerous culture media have been devised--over 7,000 were classified by Levine and Schoelein (1930)--their essential ingredients are relatively simple. Most media contain a source of carbon and nitrogen, inorganic salts, and in some cases, vitamins or other growth-promoting substances. These requirements are achieved through the use of either defined or complex media. In a chemically defined medium such as glucose-salts medium, all constituents are known and quantitated whereas in a complex media, such as meat-infusion broth, the exact chemical composition is unknown.

In defined media the nitrogen source of choice is usually ammonium salts, nitrates or urea. The suitability of these materials as compared with a complex nitrogen source (casein hydrolyzate) to support growth of *Candida utilis* is shown in Figs. 8 - 13. The most obvious observation is that *Candida utilis* growth is influenced only slightly by increased concentrations of ammonium sulphate, urea and casein hydrolyzate (Figs. 8 - 10); nevertheless, casein hydrolyzate produced earlier and faster growth as compared to ammonium sulphate and urea (Fig. 11). Similar results were reported by Rose (1961) who reported that yeasts grow more rapidly in media containing amino acids (e.g., brewer's malt wort) than in media containing ammonium salts as the sole source of nitrogen. Figs. 12 - 13 show the ability of *Candida utilis* to utilize a wide variety of inorganic sources of nitrogen, especially nitrates. Rose (1961) reported that some yeasts, such as *Candida utilis*, can use nitrate as a sole source of nitrogen for growth, whereas others, like *Saccharomyces cerevisiae* are devoid of this ability. Virtanen *et al*. (1949) found that nitrate utilization by *Candida utilis* resulted in the formation of hydroxylamine and that this reacted with pyruvic acid to yield an oxime which was then reduced to alanine. The authors stated that the rate of synthesis of protein achieved in this way was as great as that using ammonia. Nutritional studies involving *Scopulariopis brevicaulis* (Morton and MacMillan, 1954) and *Neurospora crassa* (Strauss, 1951) using ammonium nitrate as a source of nitrogen demonstrate preferential utilization of ammonium ion. Nitrate utilization does not begin until virtually all the ammonium has disappeared from the medium. A pH drop is presumptive evidence for preferential utilization of the

ammonium ion. Ammonium nitrate utilization by Candida utilis and changes in pH of the growth medium are shown in Figs. 12 - 13. In general, nitrite is only utilized by microorganisms in alkaline media for, at acid pH values, the nitrite ion is toxic. Nitrite toxicity to Candida utilis is shown in Fig. 12.

A common factor in many complex media is the presence of protein hydrolyzates. A wide variety of sources may be used to provide protein hydrolyzates. The source of protein may originate from animals, plants or microorganisms. The following protein sources have been used in the preparation of acid or enzyme hydrolyzates used in industrial or laboratory media: casein, gelatin, keratin, groundnut, soybean, cottonseed, yeast and malt extracts. Formulation and design of microbial media involves the following considerations: cost, availability, standardization and chemical composition of medium components, available carbon and nitrogen, type of microorganism and nature of the microbial process or fermentation.

Proteins are polymers of L-α-amino (or imino) acid monomers arranged in a specific linkage. The peptide bond is an imide linkage between the α-carboxyl and α-amino groups of two amino acids, however, the possibility exists that amino acids such as lysine, glutamic acid and aspartic acid are bonded through their respective ε, γ, and β-groups, be it instead of, or in addition to the α-peptide linkage. The peptide bonds can be broken under certain conditions to yield peptides, polypeptides, peptones, proteoses and amino acids in varying proportions. With the exception of amino acids these products of hydrolysis are normally considered as classes of compounds rather than individual

substances since there exists no definite line of distinction between them. The complete or partial hydrolysis of proteins may be accomplished by the use of strong acid, strong base or proteolytic enzymes.

Strong bases have not been used extensively for the hydrolysis of proteins except in situations where it is essential to minimize destruction of tyrosine and tryptophan (Jorpes, 1932; Lugg, 1938). Protein hydrolysis on a commercial scale is most commonly effected by acids or proteolytic enzymes.

Protein hydrolyzates prepared by the use of sulphuric or hydrochloric acids are characterized by the destruction of particular amino acids, notably tryptophan, glutamine, asparagine, serine and threonine. This is not the case with enzymic digests due to the specific action of the enzymes and the mild conditions of hydrolysis (Hill, 1965). Although extensive amino acid analysis was not undertaken to determine the extent of destruction of individual amino acids, the assumption is made that destruction of amino acids (free or bound) was more extensive in the acid hydrolyzates than in the enzymic digests. Studies with synthetic and protein substrates have demonstrated the specificity of trypsin and pepsin (Fruton and Anslow, 1939; Bergman, 1942; Baker, 1951; Bovey and Yanari, 1960; Inagami and Sturtevant, 1960; Bender and Kaiser, 1962; Goldstein *et al.*, 1963). Trypsin exhibits the highest degree of substrate specificity for an endopeptidase; only those bonds involving the carboxyl groups of lysyl and arginyl residues are hydrolyzed. Subsequently, tryptic digests of proteins results in the formation of relatively large peptide fragments whose carboxyl terminal end is either lysine or arginine. Amides and esters are also hydrolyzed. In contrast,

pepsin shows fairly broad specificity for peptide bonds. Pepsin hydrolyzes peptide bonds formed by either the amino or carboxyl group of phenyl alanine, tyrosine, glutamic acid, cystine and cysteine. In addition, examples have been found of hydrolysis of bonds formed by the carboxyl group of all L-amino acids except proline, however, bonds formed by other amino acids do not appear to be hydrolyzed as readily as those of the aromatic amino acids and leucine. Pepsin is also capable of hydrolyzing ester bonds (Inouye and Fruton, 1967); in both proteolysis and esterolysis, activity is favored by hydrophobic side chains on both sides of the sensitive bond.

It is well recognized that the liberation of some amino acids from peptide linkages is faster than others; furthermore, some amino acids require longer periods of acid hydrolysis before being liberated (Block and Weiss, 1956). This is substantiated by the results in Table 16. If the amino acids were all liberated at the same rate the ratio of individual amino acids in RSOM-HA^{30} (80% hydrolysis) to RSOM-H (95% hydrolysis) would be constant. This is not the case. This could have significant effects on microbial growth if an essential amino acid was not liberated during hydrolysis. Similar results may be associated with enzymic digestion; the unique characteristic of proteolytic enzymes being their specificity in hydrolyzing peptide linkages formed by specific amino acids. The specificity of the two enzymes, pepsin and trypsin, is evident from amino acid composition of the digests (Table 16). Again, as with acid hydrolyzates, this could have implication for the growth of fastidious microorganisms. In view of the above, one might expect differences in growth response of *Candida utilis* to acid and

enzyme preparations of rapeseed oil meal. However, no clear cut differences were observed (Fig. 15). The two acid preparations gave similar results. This is due to the fact that both of the media contain appreciable quantities of free amino acids which are readily available for growth. The trypsin preparation gave more rapid and pronounced growth than either of the acid preparations even though the quantities of free amino acids in the former are less than in the latter (Table 16). This would indicate that the levels of free amino acids are not rate-limiting. On the other hand, the peptic digest of RSOM resulted in slow growth and lower cell yield than both trypsin and acid preparations. The data in Table 16 would suggest that the very low levels of some amino acids particularly lysine, histidine, threonine, serine, alanine, leucine, isoleucine and tyrosine is responsible for this depressed growth response. The low levels of these amino acids may be a result of the difference in mode of action of pepsin compared to trypsin or the lower specific activity of the purified pepsin. Although it would appear that the initial levels of free amino acids in the experimental media can have an effect on growth response, it must be recognized that other factors can play an important role. In the industrial production of amino acids, for example, the amino acid balance of the medium is very important as a result of mutual competition between amino acids in the form of feed-back inhibition and repression (Kinoshita, 1963). These mechanisms are influenced by the free amino acids and not bound amino acids. This further complicates the nutritional assessment of media in which both free amino acids and bound amino acids (peptides, polypeptides) are present.

Apart from absolute requirements, many microorganisms grow better when supplied with a mixture of amino acids, proteins or protein hydrolyzates than with simple amino acids. From extensive studies with bacteria, it has been shown that growth response to individual peptides may be less than, equal to, or greater than to an equimolar amount of the essential amino acid they supply (Kihara and Snell, 1952, 1955, 1960, 1960a; Prescott et al., 1953; Peters *et al*., 1953; Leach and Snell, 1959, 1960). These authors suggested that the three divergent growth responses result when the combined operation of transport and peptidase systems supply the essential limiting amino acid from a peptide at rates less than, equal to, or greater than those at which the free amino acid can be absorbed from the medium. Kihara *et al*. (1952a) found that some microorganisms (*Streptococcus faecalis*, *Escherichia coli*) destroy certain amino acids when these are present in excess. Under these conditions, peptides of that amino acid will display heightened growth-promoting activity because they supply the amino acid in a form not subject to destruction, but from which the growth-essential amino acid can be released continuously in the low concentrations required for protein synthesis.

Although no information exists on the relative value of amino acids, peptides and polypeptides to supply the nitrogen requirements of *Candida utilis*, yeasts in general possess only limited proteolytic activity. This is not due to lack of proteolytic enzymes but rather the failure to produce these enzymes extracellularly (Joslyn, 1955). These enzymes were studied and classified as proteases, polypeptidases and dipeptidases according to their ability to hydrolyze

their respective substrates (Willstätter and Grassman, 1926; Grassman, 1927; Grassman _et_ _al_., 1928, 1934).

From Fig. 15, it is clear that although RSOM preparations support growth of _Candida_ _utilis_, soytone, an enzymatic digest of soybean meal, gives greater cell yields. This would not appear to be related to the presence of large quantities of free amino acids as RSOM-acid hydrolyzates compare favorably in this respect (Table 16). Because of complexities noted previously regarding nitrogen requirements of microorganisms, it is not possible to explain this finding in other than general terms.

Figs. 17 - 25 illustrate an attempt to prepare media based on RSOM derivatives that would give growth response comparable to commercial formulations. Supplementation of all RSOM preparations with dextrose significantly increased cell yields compared to dextrose-free media. However, increasing dextrose concentration two-fold did not give proportional increases in cell yield. The most probable explanation of the increased cell yields is that in dextrose-free media some of the amino acids are being utilized to supply energy, and as such are not available for synthesis of cellular material.

In conclusion, the results of the present study demonstrate the ability of rapeseed oil meal preparations to be used in microbial culture media. Both digests of rapeseed oil meal, acidic or enzymic, supported growth of _Candida_ _utilis_ comparable to several commercial media formulations (Tryptic Soy Broth, Czapek-Dox Broth, Sabouraud Dextrose Broth). In addition, the utilization of rapeseed oil meal as a medium component represents a novel method of circumventing

problems associated with rapeseed oil meal (toxicity, palatability, digestibility).

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